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Begin the article with a short summary.

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Screeners Conference

Drug Research and Development Program
Division of Cancer Treatment
National Cancer Institute

Editor: John M. Venditti

May 22 and 23, 1975

Silver Spring, Maryland

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Contents

Proceedings of the Screeners Conference: May 22 and 23, 1975, Silver Spring, Md

Drug Evaluation Branch Program: Report to the Screening Contractors 1
John M. Venditti
National Cancer Institute, Bethesda, Maryland

Presumptive Screening

Statistical Properties of the L1210 Mouse Leukemia Tumor System in Primary Screening 5
S. Stone and I. Miller
Arthur D. Little, Inc, Cambridge, Massachusetts

Modified Protocol for the Testing of New Synthetics in the L1210 Lymphoid Leukemia Murine Model in the DR&D Program, DCT, NCI 15
R.I. Geran, N.H. Greenberg, M.M. Macdonald, and B.J. Abbott
National Cancer Institute, Bethesda, Maryland

New Systems Development

Preclinical Characterization of Candidate Antitumor Drugs 21
Abraham Goldin, Randall K. Johnson, and John M. Venditti
National Cancer Institute, Bethesda, Maryland

Kinetics and Tumor Models 83
Linda Simpson-Herren
Southern Research Institute, Birmingham, Alabama

Solid Tumor Animal Model Therapeutically Predictive for Human Breast Cancer 89
Daniel S. Martin, Ruth A. Fugmann, Robert L. Stolfi, and Peggy E. Hayworth
Catholic Medical Center of Brooklyn and Queens, Inc, Woodhaven, New York

Growth Characteristics and Chemotherapeutic Response of Intravenously Implanted Lewis Lung Carcinoma 111
Artemio A. Ovejera, Randall K. Johnson, and Abraham Goldin
National Cancer Institute, Bethesda, Maryland

Biologic Characteristics of Tumor Progression in the Mouse Mammary Gland 127
Daniel Medina
Baylor College of Medicine, Houston, Texas

New Models for Cancer Chemotherapy 133
S.I. Detre, A.J.S. Davies, and T.A. Connors
Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, England

Preliminary Drug Trials in a Renal Cell Carcinoma Animal Model 145
Alan M. Shefner and Marilyn Marlow
IIT Research Institute, Chicago, Illinois

Ridgway Osteogenic Sarcoma—A Promising Laboratory Model for Special Therapeutic Trials Against an Advanced-Staged, Drug-Sensitive Animal Tumor System 151
W.R. Laster, Jr
Southern Research Institute, Birmingham, Alabama

A Mouse Colon-Tumor Model for Experimental Therapy 169
T.H. Corbett, D.P. Griswold, Jr, B.J. Roberts, J. Peckham, and F.M. Schabel, Jr
Southern Research Institute, Birmingham, Alabama

Combined Modality

The Potential for Murine Tumor Models in Surgical Adjuvant Chemotherapy 187
D.P. Griswold, Jr
Southern Research Institute, Birmingham, Alabama

Cell Survival Systems for the Evaluation of Irradiation and Drug Combinations 205
Vincent H. Bono, Jr, John E. Byfield, Ronald F. Hagemann, Samuel H. Lesher, Frederick A. Valeriote, and Teresa Vietti
National Cancer Institute, Bethesda, Maryland

Intramuscularly Implanted B16 Melanoma as an Animal Model for Combined γ-Radiation Therapy and Chemotherapy Studies 215
Isidore Wodinsky, Joseph K. Swiniarski, and John M. Venditti
Arthur D. Little, Inc, Cambridge, Massachusetts

Combined Modality Therapy: Actinomycín D (NSC-3053) and γ-Radiation Against the Ridgway Osteogenic Sarcoma 225
Philip C. Merker, Isidore Wodinsky, John M. Venditti, and Joseph Swiniarski
Arthur D. Little, Inc, Cambridge, Massachusetts

Interaction of Experimental and Clinical Studies in Combined Modality Treatment 235
Stephen K. Carter and Todd H. Wasserman
National Cancer Institute, Bethesda, Maryland

Data Analysis and Processing

Management and Analysis of Mortality Data Obtained by Treating Small Experimental Animals With Anticancer Drugs 243
Harris H. Lloyd, John W. Love, and Robert D. Evans
Southern Research Institute, Birmingham, Alabama

Impact Upon Screeners of the Front-End Redesign 253
William T. Miller, Jr
Value Engineering Co, Alexandria, Virginia

The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1976.

Drug Evaluation Branch Program: Report to the Screening Contractors

John M. Venditti[1,2]

The mission of the Drug Evaluation Branch (DEB) of the DR&DP, DCT, NCI, is to develop and use laboratory models for the identification, evaluation, and prediction of new therapies for the control and cure of human malignant disease. Selection of screening assays to properly evaluate the thousands of synthetic and natural products submitted to the DR&DP is crucial to the successful attainment of this goal. Ideally, with respect to initial screening for antitumor activity, one would wish to positively identify one or a few in vivo or in vitro (including biochemical) assays that would predict for clinical activity absolutely. Despite the emergence of laboratory models with a high degree of predictive value (1,2), no single model or combination of models can be said, at this time, to predict perfectly for therapeutic efficacy in man. Alternatively, one might wish to test all new materials in a wide spectrum of assays including those known to correlate well and those which have been rationally selected but are as yet unevaluated. With respect to in vivo screens, past attempts to approach this alternative were hindered by the small amounts of many of the compounds submitted to the screen and by a limited capacity. The problem has been intensified in recent years by an increased input of materials as well as by attempts to maintain screening capacity at reasonable levels in the face of cost inflation. Consequently, it had been necessary to select one model for initial screening. Wider spectrum screening has been reserved for materials that are "active" in our primary screen or other screening programs, and for compounds of special interest based on the rationale for their synthesis. Primary screens have been selected because of their sensitivity to agents of known clinical value, and secondary screens because they exhibit some biologic characteristic different from those of primary screens, eg, tumor cell growth kinetics or histopathologic resemblance to specific human tumors. An obvious disadvantage to this approach is the requirement that randomly acquired materials must "pass" a limited primary screen to be candidates for wider spectrum evaluation. Thus, the desirable attributes of a large-scale primary screen are as follows: predictability of a maximum number of true-positives and a minimum number of false-negatives for the clinic; reproducibility; feasibility for testing a large number of limited-quantity materials; rapid elimination of inactives; and high cost efficiency so that more resources can be applied to secondary screening and detailed drug evaluation.

Critical evaluation of a number of transplantable animal tumors has led to our current use of the L1210 and P388 leukemias for primary screening of random synthetic compounds and crude natural products respectively. L1210 is used because of its demonstrated sensitivity to most clinically active drugs (1,2) and P388 is used because it is quantitatively more sensitive than L1210 (2). If initial screening for most of the materials submitted to this program must be restricted to one system (in 1974, 75% of acquired synthetic materials were received in an amount of 2.0 g or less), and presuming that there will be a broadening of the number of systems used for secondary screening to provide a further basis for selection of drugs for priority development, an initial screen of greater sensitivity than L1210 might be considered. P388 appears to be as predictive as L1210 for agents active against human tumors and is more sensitive than L1210 to the clinically useful representatives of most major classes of antitumor drugs (2). Other models of even greater sensitivity should be investigated. Such a system, in addition to being rapid and relatively inexpensive, should select a high percentage of true-positive drugs for the clinic. Selection of a high number of *potential* false-positives might be expected but would not necessarily be undesirable if a substantial screening spectrum were used to evaluate them further. Priorities for development could be based on spectrum of activity and degree of activity in sensitive systems.

The possibility of using rapid and relatively inexpensive cell culture, biochemical, or antimicrobial assays for initial screening has been considered. In the DEB screening program, a cell culture (KB) screen is routinely used, along with the in vivo P388 screen, for initial testing of crude natural products. Samples which show correlative activity in both systems are fractionated using the less expensive KB system to bioassay the activity of the fractions. KB is also used to test synthetic materials received in very small amounts and for selected other synthetics of interest. Use of this cell culture model as a pre-screen would tend to select a population of compounds that would yield a higher percentage of in vivo actives than would de novo in vivo screening. However, a recent retrospective study has shown that if used by our current methods, this screen would have missed a substantial number of drugs active in both animals and man. Of 2022 KB actives

[1]Drug Research and Development Program (DR&DP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, Md.

[2]*Reprint requests to:* John M. Venditti, PhD, DR&DP, DCT, NCI, 8300 Colesville Rd, Blair Bldg, Rm 532B, National Institutes of Health, Silver Spring, Md 20910.

that were tested in vivo, 313 (15%) were active in at least one in vivo system. However, 451 compounds negative against KB, including a number of clinically active drugs, showed confirmed activity against at least one animal tumor in vivo (fig 1). Of course, in vitro as well as in vivo results are dependent on the cell line, the experimental method, the parameter of growth inhibition, and the quantitative cut-off for "activity." The analysis was based on the arbitrarily chosen cut-off for KB activity of an ED50 of 4.0 μg/ml. We have not looked at the correlations that would result if we were to increase it, for example, to 10 or 100 μg/ml. Even if we could establish a cut-off that would select most of the potential clinical actives, the screen might select a high percentage of the total materials screened and still miss a few very important drugs that require metabolic activation. Comprehensive results on activity in other mammalian cell culture screens, including those that might utilize microsomal enzymes, are not available for a sufficient number of compounds evaluated in animals to permit such retrospective analyses. Prospective analyses of correlations among alternative cell culture screens and animal screens would require considerable basic and developmental investigation.

Biochemical models are used in this program to study the intracellular actions of drugs that are currently in the clinic or are in development toward clinical trial and to determine the degree of similarity of biochemical sites of action among in vivo active structural congeners. Models are designed individually to measure actions against precise stages of the cell life cycle, biochemical pathways, or target enzymes. They are useful for determining the ability of a designed compound to elicit the action proposed and, if the compound shows even minimal antitumor selectivity, this could lead to profitable new synthetic approaches. However, the use of any single biochemical end-point for large-scale screening would severely restrict the kinds of active compounds detected. A spectrum of broad biochemical screens (eg, to detect DNA, RNA, and protein inhibition) used as pre-screens would tend to circumscribe a population of agents containing a relatively high percentage of in vivo actives, but might tend to compromise our ability to detect new antitumor drugs with important, but as yet unknown, mechanisms of action. Increasing the number of test systems to include all novel approaches that emerge from basic studies might be highly productive, but might also substantially increase screening costs. Antimicrobial screens are used to study patterns of cross-resistance among clinically active drugs and their structural congeners. In addition, drugs in development for the clinic are tested against a spectrum of microorganisms to provide a bioassay for determining the in vivo physiologic distribution and effective duration of drug activity. The use of an antimicrobial model as a pre-screen for antitumor activity seems hazardous for a number of reasons including the problem of drug transport across a cell membrane so different from that of mammalian cells. Thus, at this time (and I wish to emphasize "at this time") we have no data that dispute the earlier conclusion of Gellhorn and Hirschberg (3) that "there is no evidence for the existence of any nontumor system which could replace a tumor system as a screening tool for carcinostatic agents." This is particularly applicable to the problem of "random" screening where one must test a large number of diverse materials for which there is no prior indication of biologic activity—either real or imagined. Materials which may be new, but are nevertheless designed on the basis of an experimentally valid rationale, present a different question. If these agents demonstrate the proposed experimental activity in vitro, they should be accorded the broadest possible in vivo testing, especially in animal tumor assays designed to take advantage of the known actions of the material.

Historically, many drugs have entered clinical trial on bases other than the limited primary animal screens that have been used in this program. These have included reports of activity in the clinic, in animal screens from other programs, in in vitro systems, in endocrine systems, and in immune response screens. It should not be inferred from this commentary that alternative biologic, biochemical, or pharmacologic activities should not form the basis for consideration for clinical trial. However, in our opinion, regardless of the genesis of a new agent, unless it is shown to affect some factor unique to the human host-tumor relationship (and consequently not evaluable in an animal system), its ability to elicit a therapeutic response or to potentiate the efficacy of an existing therapy in some animal system should be a prerequisite for its clinical trial. This presupposes the widening of the spectrum of in vivo models to include diverse transplantable, spontaneous, and virus-induced tumors which exhibit different growth, biochemical, histopathologic, and immunologic characteristics of known or potential importance, and to include biologically characterized animal models for specific human neoplasms. A substantial part of this

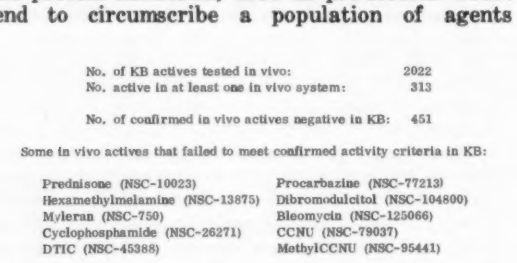
No. of KB actives tested in vivo: 2022
No. active in at least one in vivo system: 313
No. of confirmed in vivo actives negative in KB: 451
Some in vivo actives that failed to meet confirmed activity criteria in KB:
Prednisone (NSC-10023)
Hexamethylmelamine (NSC-13875)
Myleran (NSC-750)
Cyclophosphamide (NSC-26271)
DTIC (NSC-45388)
Procarbazine (NSC-77213)
Dibromodulcitol (NSC-104800)
Bleomycin (NSC-125066)
CCNU (NSC-79037)
MethylCCNU (NSC-95441)
A material was considered "active" against KB if it produced a growth inhibitory ED50 at a concentration ≤ 4 μg/ml.

FIGURE 1.—Relationship of activity against KB cells in culture and activity against animal tumors in vivo applying current criteria for activity.

Screening Symposium has been devoted to the development of several models which are in current limited use. In addition to the potential usefulness of such models for secondary screening, combination chemotherapy, and combined modality studies, we should address ourselves to the question of whether some could be used as prospective primary screens for at least some new agents. In reducing the question to its simplest terms we can ask whether we should try to set up the "ideal" broad-spectrum primary screen or pursue the "sensitive pre-screen" approach (fig 2). The main advantages and disadvantages of these extremes, as I see them, are summarized in figures 3 and 4. At present we are exploring the feasibility of alternative 3 in figure 5. Successful implementation and conduct of this plan would require the DR&DP to limit the number of materials for its pre-screen to facilitate the expanded use of a secondary spectrum of models. We expect to accomplish this by practicing greater selection in solicitations of random materials. A means of comprehensive surveillance of the scientific literature and other screening programs throughout the world would need to be established. Candidate agents for secondary screening should be tested in all of the assays comprising the spectrum. If materials emerging as active from any of the spectrum assays were accorded adequate clinical trial, a number of important questions could be answered with respect to the selection of new anticancer drugs and screening models as well. Such a plan would be expected to provide critical information relative to the value of human tumor xenografts versus spontaneous animal tumors versus transplanted animal tumors of similar histopathologic type. Two comprehensive retrospective analyses (1,2) have indicated that the probability of clinical activity for a drug increases as the spectrum of animal tumor sensitivity increases. Successful implementation of alternative 3 in figure 5 would provide a prospective test of the reality of this indication.

1. Set up an "ideal" screen, ie, prospectively screen all submissions in spectrum of models. For example:

   L1210
   Mammary carcinoma in mice (spontaneous and transplanted)
   Colon tumors in mice (carcinogen-induced and transplanted)
   Xenografts of human tumors grown in mice
   Ridgway osteogenic sarcoma in mice
   Hyperplastic alveolar nodule in mice
   Lung squamous cell carcinoma in mice
   Renal cell carcinoma in mice
   Other organ and tissue specific tumors
   One or more in vitro screens

2. Use a very sensitive "pre-screen" designed to pick up any material that would be active against one or more of the "tumor spectrum."

   In vivo
   Mammalian cell cultures
   Biochemical screens
   Antimicrobial screens

FIGURE 2.—Two proposed alternatives to the current screening practice.

1. Advantages (presuming clinical trial of any material showing activity in any system)

   a. Probably little chance of missing a clinically active drug

   b. In time, would provide a basis for prospective analysis of value of each model or combination of models

   c. Would permit comparison of each system with L1210 for predictive value

2. Disadvantages

   a. Cost of screening each material would increase markedly

   b. Pragmatically, only materials received in very large amounts could be completed unless program was prepared to synthesize materials with no previous indication of activity

   c. Finding enough "actives" to evaluate each system would require considerable time

   d. Time to clinical evaluation would take more than 3 years

      From DN-2A to phase I clinical trial, 1-2 years
      Completion of phase I clinical trial, 1 year
      Completion of phase II clinical trial, ?

FIGURE 3.—Advantages and disadvantages of broad-spectrum initial screening.

1. Advantages

   a. Could maintain high input

   b. Eliminates inactives quickly and cheaply

   c. Requires minimal amount of material

2. Disadvantages

   Still would present a "barrier" to finding unique activity inasmuch as it represents a preselection based on demonstration of some specified biologic activity

FIGURE 4.—Advantages and disadvantages of the pre-screen approach.

1. Limit screening input to those materials that can be placed initially in broad-spectrum screening based on amount received and current screening capacity.

2. Use a very sensitive, but reasonably discriminating "pre-screen" for initial screening and depend on it exclusively to select materials for "broad-spectrum" screening.

3. Select materials for "broad-spectrum" screening from:

   a. The DR&DP "pre-screen";

   b. Other antitumor screening programs;

   c. Consideration of certain reported biologic and biochemical activities as delineated in the world's literature.

FIGURE 5.—Alternative approaches to screening.

We will continue the detailed evaluation of drugs in development toward clinical trial, as single agents and in combinations, with respect to those factors known to affect therapeutic efficacy such as route and treatment schedule, the total tumor cell burden when treatment is begun, the tumor implant site, etc. Schedule-dependency tests should continue to include parallel groups of tumor-bearing and

normal mice. The possibility of estimating starting doses in man from toxicity data in mice (4) is intriguing. The computer program in use at the Southern Research Institute to estimate the LD10 and LD50 from the laboratory's total normal mouse experience for a drug provides an important step in pursuing this possibility. Extension of this type of mortality data analysis to all normal animal toxicity data generated in this program would be highly desirable.

In recent years, the DR&DP has initiated a number of new contracts devoted to fundamental aspects of drug action and has continued its related intramural studies. Specifically, these objectives are aimed at drug effects on growth kinetics of normal and tumor cells (in vivo and in vitro) and elucidation of cellular sites of action of drugs in development toward clinical trial or of special interest. During the next year, increased emphasis will be placed on the development and use of models for the evaluation of combined treatment modalities. As discussed by Carter and Wasserman (5), the DCT has identified, as a major approach to improving cure rates among human solid tumors, the integration of chemotherapy with local modality therapy, ie, surgery or radiation.

Routinely, new structural congeners of clinically active drugs are examined in in vitro and in vivo systems selected on the basis of the characteristics of the parent drug. Objectives of this project are to determine whether the new active congener differs from the parent with respect to its biologic, biochemical, or pharmacologic action and thus should be considered a unique agent; to provide rapid feedback of information to synthetic chemists to aid the direction of synthesis; and to determine potential therapeutic superiority over the parent drug. The ultimate success of this program depends on (a) identification of structural classes which should be of interest to the program; (b) selection of in vivo screens including drug-resistant variant tumors as well as in vitro and biochemical screens that will aid in determining whether the congener is a true pharmacologic analog of the parent compound; and (c) establishment of assay systems and criteria to judge the potential superiority of the congener (ie, remarkably higher activity in a test system, wider spectrum of activity, lack of cross-resistance, other indications of a difference in biochemical or pharmacologic action, diminution of a specific toxicity that limits the clinical usefulness of the parent drug, or greater ease of acquisition or formulation for the clinic). We might further improve our evaluation of new structural congeners of known antitumor drugs by fostering an even greater degree of direct communication and collaboration among the chemists who synthesize new drugs, the DR&DP contractors who evaluate them, and other areas of the DCT that may be in a position to conduct crucial comparative studies (eg, toxicology) outside the present scope of DR&DP activities.

An outline of what we believe to be a comprehensive strategy for proper preclinical evaluation of new drugs emerging from primary screens is discussed in the commentary by Goldin et al (6) presented in this issue.

As we accumulate the results of additional studies of individual drugs, drug combinations, and combined modalities from diverse laboratory models and from the clinic, it will be desirable to determine which combinations of in vivo and in vitro screens predict best for the clinic in general and for the therapy of particular human cancers. The DEB, in collaboration with its contractors and the Investigational Drug Branch, Cancer Therapy Evaluation Program, DCT, has been extending the analysis of both animal and human data to develop a more accurate picture of the relationship of clinical results to screening. During the next year, more emphasis should be placed on correlations between activity in individual experimental models and usefulness against specific human tumors. Hopefully, on the basis of more quantitative information, both clinical and animal, prediction formulas for establishing priorities for the development of compounds toward clinical trial will be refined and will permit the inclusion, into the overall evaluation, of results from new in vivo and in vitro systems along with results from current systems.

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Statistical Properties of the L1210 Mouse Leukemia Tumor System in Primary Screening[1]

S. Stone and I. Miller[2,3]

SUMMARY

Statistical analyses on the results of an experiment comparing the ability of three- and six-animal test groups to identify and confirm active compounds led to the following recommendations: *(a)* random synthetic compounds should be initially tested in three-animal test groups and declared "active" if the "optimum" T/C value is 120 or greater; and *(b)* confirmation testing should be performed using six-animal test groups. A quality-control strategy for the L1210 tumor system is recommended.

[Cancer Chemother Rep Part 2, vol 5:5-13, 1975]

The L1210 mouse leukemia tumor system has been the principal animal model for primary screening of synthetic potential anticancer compounds for several years (1,2). An important basis for the choice of this system was its reproducibility; untreated (control) BDF_1 or CDF_1 mice inoculated intraperitoneally (ip) with 10^5 L1210 leukemia cells generally die between the 8th and the 10th day after inoculation. In addition, experience with highly active compounds (positive controls) has shown good reproducibility in the ratio of mean survival times for treated groups to those for associated control groups (T/C values).

The protocol for the screen specifies six-animal treatment groups and associated control groups of approximately 30 animals for use in dose-response testing (3). A compound was said to be "presumed active" if, for some dose in the nontoxic range, the ratio

$$\frac{T}{C} = \frac{\text{mean survival time of treated animals}}{\text{mean survival time of control animals}} \times 100$$

exceeds 125, corresponding to a 25% increase in lifespan (ILS) (4). Subsequently, in an effort to deal with the problem of compounds not available in sufficient quantity for six-animal testing and to reduce screening costs, the number of test-group animals was reduced to three and the number of control-group animals was reduced correspondingly.[4] This new "miniscreen" protocol was selectively introduced into the screen for use with compounds in small quantities in May 1973.

After about 1 year's experience with the miniscreen (June 1974), the Screening Section of the Drug Evaluation Branch, Drug Research and Development, Division of Cancer Treatment, NCI, designed a protocol for the purpose of prospectively comparing its screening results with those that would have been obtained if the six-animal screen had been used. Under this protocol, seven screening laboratories have tested a total of 2163 random synthetic compounds. The data generated by this experiment have provided an unusual opportunity to study the statistical properties of the L1210 leukemia screen. It has become feasible to re-examine the choice of a boundary value of T/C for presumed activity ("cutoff" value), to compare the variability of results in three- and six-animal test groups, and to establish "quality-control" limits for use with control-group survival-time distributions.

Outline of the Protocol

The protocol is described schematically in figure 1. Seven screening laboratories participated in the experiment: Hazleton Laboratories; Battelle Memorial Institute; WARF Institute, Inc.; Arthur D. Little, Inc.; Southern Research Institute; IIT Research Institute; and Mason Research Institute.

Each screener tested numerous synthetic compounds, using both three- and six-animal test groups. The four steps in figure 1 are therapy regimens which were used sequentially as follows: Days 2 and 6 (step 1); Days 1-9 (step 2); Day 1 only (step 3); and Days 1, 5, and 9 (step 4).

[1]Supported by contract N01-CM-33727 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[2]Arthur D. Little, Inc., Cambridge, Mass.

[3]*Reprint requests to:* Ms. Sandra H. Stone, Arthur D. Little, Inc., Acorn Park, Cambridge, Mass 02140.

[4]Goldin A. The concepts of the mini and the econo screen. Report of the Division of Cancer Treatment, NCI, 1973. Bethesda, Md, NCI, 1973, vol 2, pp 2.76-2.99.

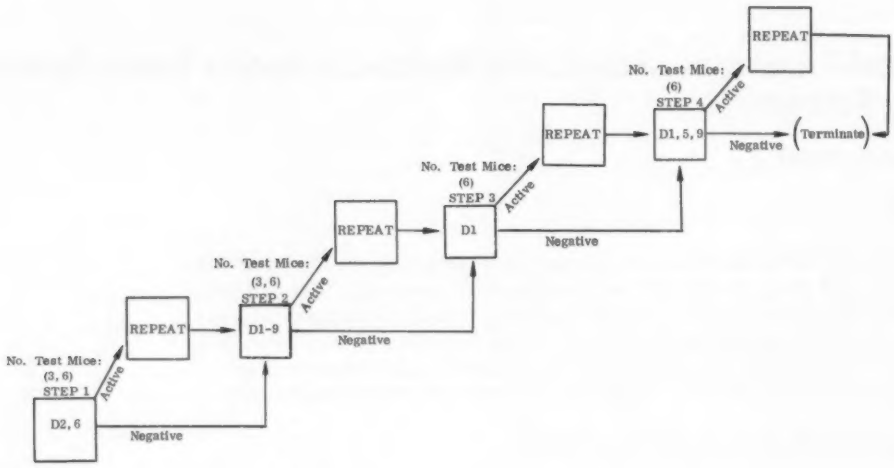

FIGURE 1.—Experimental design of the protocol.

Subject to the availability of the compound, step 2 was performed using both three- and six-animal test groups; steps 3 and 4 were performed using six-animal test groups only. If there was an insufficient quantity of drug to perform step 2 completely, the laboratory omitted steps 2 and 3 but performed step 4 with each test-group size. If sufficient compound then remained, step 3 was performed with each test-group size.

The protocol specified that if a compound was found to be "presumed active" (T/C value of at least 125) in any given regimen for either the three- or the six-animal test group, *both* tests (three and six animals) were to be repeated in that regimen.

Initial and Confirmed Activity

A compound was said to be an "initial active" if the largest T/C value resulting from the first non-toxic dose-response test ("optimum" T/C) exceeded 125. If after repeating the test with the same test-group size (three or six animals) the optimum T/C value again exceeded 125, the compound was said to be a "confirmed active."

The extent of initial activity found is summarized in table 1. Note that the proportions of initial actives identified were from 2½ to 7 times greater for three-animal test groups than for six-animal test groups, depending on the therapy regimen. This kind of result was not unexpected on purely statistical grounds. The distribution of T/C values on repeat testing is well represented by the log-normal distribution, having a long right-handed "tail." The theory of sampling variability leads us to conclude that the length of the right-hand tail will be greater for smaller test-group sizes, thus favoring more values of T/C in excess of 125.

Of the compounds tested, six were found to be "confirmed actives" in at least one of the four test schedules (table 2). NSC-195458 and NSC-210924MA00 were initially inactive in the six-animal test groups in the three therapy regimens not shown in table 2, and they were untested in the three-animal test groups. NSC-211519 was initially inactive in the three remaining treatment schedules for both three- and six-animal test groups not shown. NSC-218476MA00 and NSC-214541MA00 were initially inactive in the therapy regimens not shown.

TABLE 1.—Extent of initial activity

Therapy regimen	3-Animal test groups			6-Animal test groups		
	No. of compounds tested	No. of initial actives	%	No. of compounds tested	No. of initial actives	%
Days 2, 6	1943	57	2.9	2005	8	0.4
Days 1-9	1618	24	1.5	1697	8	0.5
Day 1 only	313	5	1.6	1892	7	0.4
Days 1, 5, 9	380	7	1.8	1836	13	0.7

TABLE 2.—Six compounds showing confirmed activity

NSC No.	Therapy regimen	No. of animals in test group	Optimum T/C values		Remarks
			Initial test	Repeat	
195458	Days 2, 6	3	129	127	Confirmed active
		6	125	112	Unconfirmed active
210924MA00	Days 1-9	3	125	160	Confirmed active
		6	127	136	Confirmed active
211519	Days 1, 5, 9	3	132	126	Confirmed active
		6	117	116	Inactive
218441MA00	Days 2, 6	3	245	322	Confirmed active
		6	140	300	Confirmed active
	Days 1-9	3	336	329	Confirmed active
		6	308	322	Confirmed active
	Day 1 only	6	215	181	Confirmed active
	Days 1, 5, 9	6	354	178	Confirmed active
218476MA00	Days 1-9	3	126	114	Unconfirmed active
		6	128	134	Confirmed active
	Day 1 only	6	129	117	Unconfirmed active
214541MA00	Days 2, 6	3	142	181	Confirmed active
		6	108	115	Inactive

From table 2 we can observe that the three- and six-animal test groups yielded seemingly contradictory results in four of the seven cases in which comparison was possible. NSC-195458 showed confirmed activity in the three-animal test group at a T/C level less than 130, and showed initial activity in the six-animal test group (T/C = 125) which failed to confirm. NSC-211519 showed confirmed activity in the three-animal test group at a T/C level less than 135, and showed no activity with either test in the six-animal test group. NSC-218476MA00 showed initial activity in the three-animal test group (T/C = 126) which failed to confirm, but showed confirmed activity at a T/C level less than 135 in the six-animal test group. Finally, NSC-214541MA00 showed confirmed activity in the three-animal test group at a moderate T/C level, but showed no activity with either test in the six-animal test group.

In spite of the fact that over 2000 random compounds were tested, only six showed confirmed activity in one or more of the four treatment regimens. Of these, only one compound (NSC-218441MA00) showed a high level of activity in all four regimens and in both test-group sizes. Although there seems to be a slight tendency for the six-animal test groups to yield fewer confirmed actives than the three-animal groups, there is insufficient evidence for a meaningful *direct* statistical comparison of confirmation results relative to the size of the test group. There were, however, over 100 repeat-test pairs for each size test group using the Days-2-and-6 and the Days-1-9 therapy regimens. These data provide a uniquely available basis for a study of the reproducibility of the L1210 leukemia tumor system under various therapy regimens and with both three- and six-animal test groups.

OPERATING CHARACTERISTICS OF THE SCREEN

The primary screen can be regarded as a continuous, ongoing test of hypotheses concerning the activity of compounds submitted for screening. In the language of the statistical theory of hypothesis testing, the screen is a process for testing the null hypothesis

H_{NULL}: the compound is inactive against the given tumor model

against the alternative hypothesis

H_{ALT}: the compound is active against the tumor model.

Ideally, activity is defined as *any* true enhancement of life expectancy under some "optimum" combination of dose and regimen. In actual practice, ac-

tivity must be inferred from nontrivial lifetime enhancement, measured in terms of the parameter T/C, corresponding to at least one of the combinations of dose and regimen actually employed.

If it can be assumed that the doses actually employed "cover" the optimum dose for any given regimen, these hypotheses can be translated into the

$$H_{NULL}: \text{ optimum T/C} = 100$$

$$H_{ALT}: \text{ optimum T/C} > 100$$

for any given regimen. In practice, the test is conducted by choosing a value of T/C greater than 100, the "cutoff value," and concluding that the compound is "presumed active" if

$$\text{optimum T/C} \geq \text{cutoff T/C}$$

and "presumed inactive" otherwise.

This procedure, of course, is subject to errors arising from biologic and other sources of experimental variability. The types of errors that can be made are summarized in figure 2.

	EXPERIMENTAL RESULT	
TRUE SITUATION	Optimum T/C ≥ cutoff T/C	Optimum T/C < cutoff T/C
Compound is active	No error	False negative
Compound is inactive	False positive	No error

FIGURE 2.—Possible errors in screening.

The "goodness" of any screening procedure can be measured by the frequency with which it makes errors of these two kinds, that is, the probabilities of false negatives and false positives. To examine this important idea in greater detail, suppose the cutoff value of T/C, chosen as the basis for an activity decision, is denoted by K. The probability that the test will result in an initial active depends on the true (unknown) optimum T/C value as follows:

$$\Pr[\text{initial active}] = \Pr[\text{optimum T/C} > K, \text{ given true optimum T/C} = x].$$

A graph of this probability as it varies with values of x, the true optimum T/C, is called the *operating characteristic curve* (OC curve) of the test, and it completely specifies the frequencies with which the screen will make errors of the two kinds described. An "ideal" OC curve, one that corresponds to a situation having no experimental error, is shown in figure 3. Such a test will produce neither false positives nor false negatives; it will sort out incoming compounds into active and inactive categories without error.

In practice, because of the unavoidable presence of experimental error, the OC curve will be more like the one shown in figure 4. Note that we can readily identify the probability of a false positive as shown, because the null hypothesis is a point hypothesis—it completely specifies the true value of the optimum T/C that should lead to rejection. In order to identify the probability of a false negative, however, we need first to choose a value of T/C such that it is critical to identify as being active any compound having a true T/C value that large or larger. For example, we may identify 150 as the critical value, arguing that it is not too serious a matter if we reject a compound with true activity less than 150, but we will become concerned if a compound with a true T/C value of 150 or greater is declared "inactive." In that case, the probability of a false negative is given by the probability that a compound with a true T/C value equal to 150 will have an optimum T/C value greater than K. This probability equals 1 minus the ordinate of the OC curve at x = 150.

Actual values of probabilities on the OC curve can be calculated from data derived from the current experiment. The statistical theory underlying these

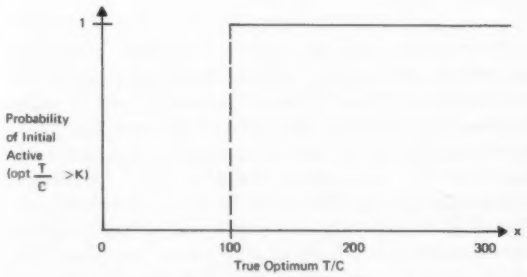

FIGURE 3.—Ideal OC curve.

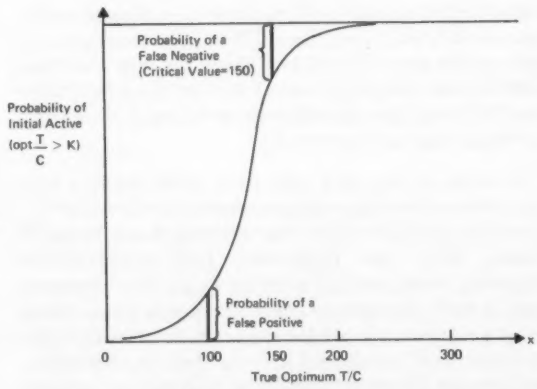

FIGURE 4.—OC curve.

calculations assumes only that the natural logarithms of experimentally determined values of T/C have the normal distribution whose mean value is the natural logarithm of the "true optimum T/C" and whose standard deviation depends only on the therapy regimen and the test-group size specified (including the control-group size associated with that test-group size.)

The data of the experiment afford an excellent and unparalleled opportunity to measure the standard deviations of ln T/C on repeat testing. Table 3 shows that six-animal test groups lead to more reproducible results than do three-animal test groups. The squares of the ratios of their standard deviations

$$\left[\left(\frac{0.109}{0.070}\right)^2 \text{ and } \left(\frac{0.107}{0.064}\right)^2\right]$$

are somewhat greater than 2, the value that would have been predicted by naive statistical theory. This appears to indicate that the "effective" ratio of sample sizes is somewhat greater than 2:1 (2.4 and 2.8, respectively) when one also takes into account differences in the associated control-group sizes.

Table 3 also shows that the first two therapy regimens are more reproducible (have smaller standard deviations) than the remaining two regimens for six-animal test groups, and that their reproducibilities do not differ materially from one another for comparable test-group sizes. In particular, the reproducibility of the Day-1-only regimen is especially poor when compared to that of the other schedules.

OC curves have been developed for each of the six combinations of therapy regimen and test-group size whose standard deviations have been calculated. Figure 5 shows the OC curves for the three- and six-animal test groups receiving the Days-2-and-6 therapy regimen that correspond to a T/C cutoff value of 125. The OC curve for the three-animal test groups is somewhat "flatter" (less like the ideal OC curve) than the curve for the six-animal test groups. Nevertheless, both sizes of test group yield excellent operating characteristics. The probability of a false positive is 0.001 for six-animal test groups and is 0.020 for three-animal groups, while the probabilities of false negatives at a critical true T/C of 150 are 0.005 and 0.047, respectively. Corresponding probabilities for other combinations of regimen and test-group size are shown in table 4. This table shows that, except for the Day-1-only therapy regimen, the probability of "missing" a compound on initial testing whose true activity level (as measured

TABLE 3.—Reproducibility of the L1210 leukemia test screen

	SD of ln T/C			
	3-Animal test groups		6-Animal test groups	
Therapy regimen	No. of repeat tests	SD	No. of repeat tests	SD
Days 2, 6	140	0.109	170	0.070
Days 1-9	110	0.107	146	0.064
Day 1 only	9	*	38	0.156
Days 1, 5, 9	16	*	56	0.100

*SDs based on sample sizes < 30 would have provided unreliable estimates and they were not calculated.

TABLE 4.—Error probabilities associated with the L1210 leukemia test screen (initial tests)

Therapy regimen	No. of animals in test group	Probability of false positive	Probability of false negative for a critical T/C of—	
			150	180
Days 2, 6	3	0.020	0.047	0.000
	6	0.001	0.005	0.000
Days 1-9	3	0.018	0.044	0.000
	6	0.000	0.002	0.000
Day 1 only	6	0.076	0.121	0.011
Days 1, 5, 9	6	0.013	0.034	0.000

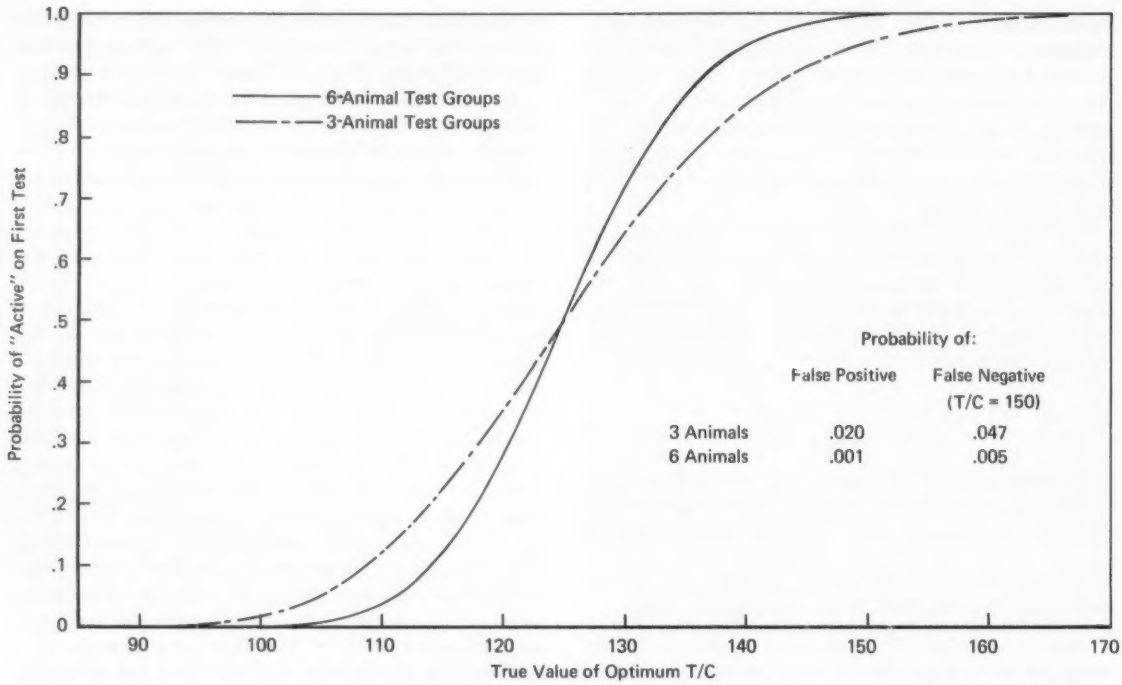

FIGURE 5.—OC curves for the three- and six-animal test groups on the Days-2-and-6 therapy regimen that correspond to a T/C cutoff value of 125; initial actives.

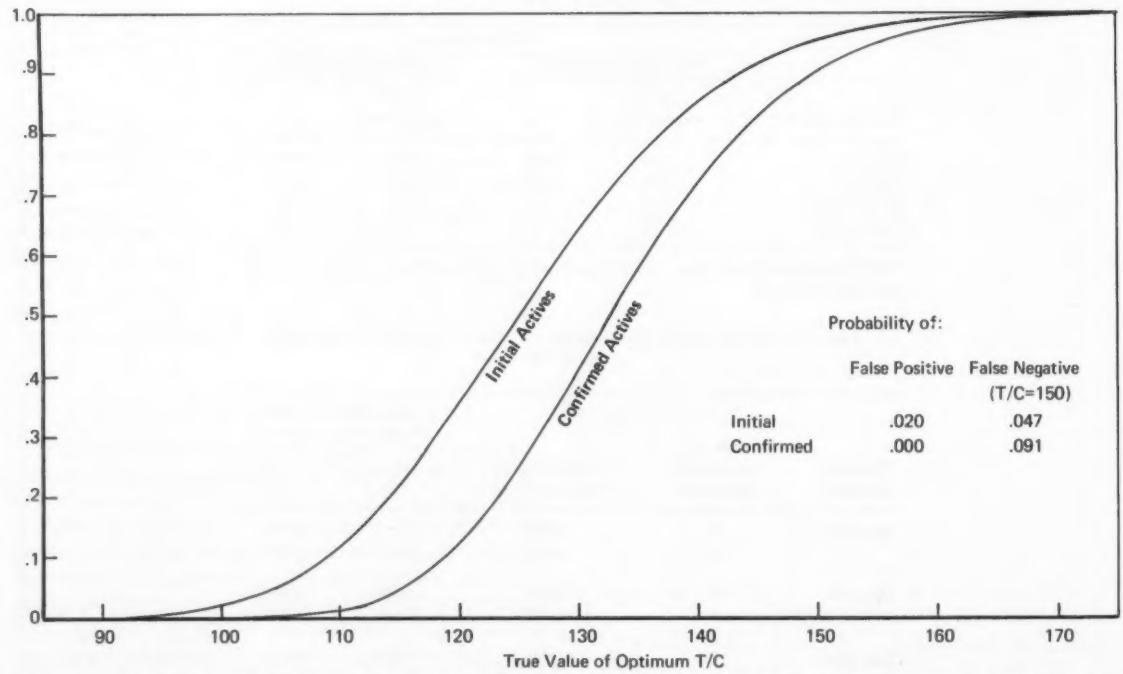

FIGURE 6.—OC curves for both initial and confirmed activity for the three-animal test groups in the Days-2-and-6 therapy regimen; T/C cutoff value of 125.

by optimum T/C) is 180 or greater is insignificant, even when three-animal test groups are used.

SOME RECOMMENDED CHANGES IN SCREENING STRATEGY

Statistical theory leads to the conclusion that the probability of obtaining a confirmed active is the square of the corresponding probability of obtaining an initial active. It is implied by this conclusion that the probability of a false positive will decrease sharply under confirmation testing (in which a compound is regarded as being presumed active only if it appears active on two successive independent dose-response tests), and the probability of a false negative will increase less markedly in proportion to its original value. This phenomenon is illustrated in figure 6, showing the OC curves for both initial and confirmed activity for three-animal test groups in the Days-2-and-6 therapy regimen. Note that the probability of a false positive decreases from 0.020 for initial testing to 0.000 for confirmation testing, while the probability of a false negative (at a critical level of 150) increases from 0.047 to 0.091.

These results show that the probability of a *confirmed* false positive is virtually zero. At the cost of increasing this probability only slightly, it is possible materially to reduce the probability that an active compound will not be confirmed as being active.

This result can be achieved by changing the cutoff value of T/C from its present 125 to some lower value, such as 120. The OC curves associated with such decreases in the T/C cutoff value are shown in figure 7. From this figure we observe that the probability of a confirmed false positive in three-animal testing increases from 0.000 to 0.002, but the probability of a confirmed false negative (at a critical level of 150) drops from 0.091 to 0.040.

Since the initial test removes the overwhelming majority of random compounds from further testing (in the present experiment, only 2.9% of the compounds tested were initially active in the three-animal screen), little additional cost would result from confirmation testing using six-animal test groups. The OC curve corresponding to initial testing with three-animal test groups and confirmation testing with six-animal test groups (using a cutoff of 120 for both tests) is also shown in figure 7. It is evident from this curve that the probability of a false positive would drop from 0.002 to 0.000 and the probability of a false negative (at 150) would decrease from 0.040 to 0.021 under this scheme.

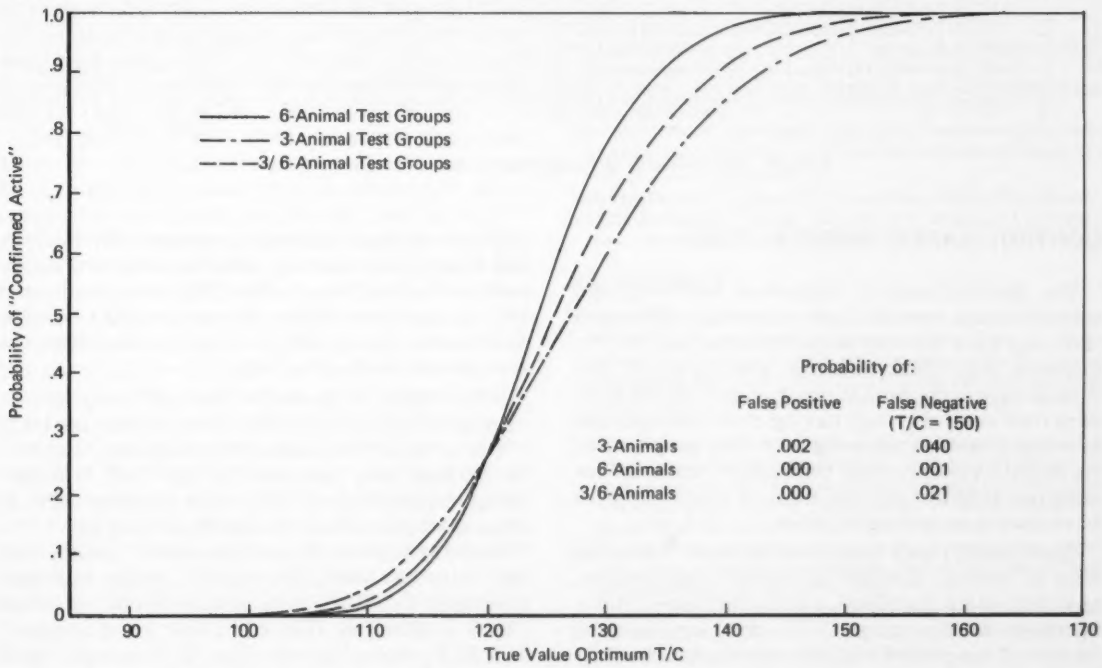

FIGURE 7.—OC curves associated with a decrease in the T/C cutoff value from 125 to 120 in the Days-2-and-6 therapy regimen for confirmation testing.

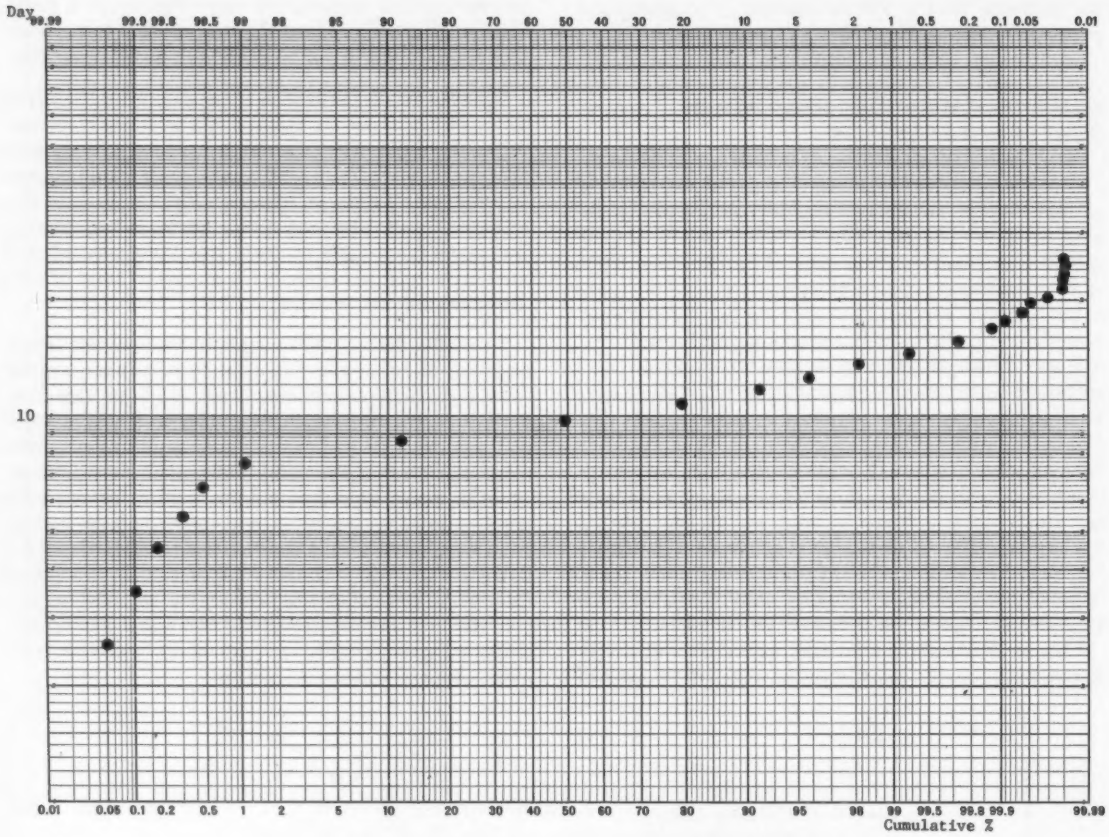

FIGURE 8.—Distribution of BDF_1 individual control-animal survival times.

CONTROL-ANIMAL SURVIVAL TIMES

The distributions of individual control-animal survival times provide clues concerning differences that may exist between animal sources and the frequencies with which abnormal results occur. The distribution of the days to death of over 39,000 BDF_1 mice from control groups having control status code 1 (acceptable) is shown in figure 8. This graph exhibits definite evidence that contradicts the usual assumption that the survival times of control animals have the log-normal distribution.

Since such a result could have resulted from a mixture of several distinct log-normal distributions, that part of the distribution shown in figure 8 corresponding to the range 7-16 days, representing 99.44% of the control animals, was replotted on log-probability paper, with a result in excellent agreement with log-normal theory. The data points in the 0-6-day and 17-29-day ranges were separately plotted with results in general agreement with log-normal theory, but showing different standard deviations as well as mean values. This work, replicated with analogous results for data relating to over 7600 CDF_1 mice, led us to divide control animals into the four groups shown in table 5.

With respect to the 39,360 "normal" tumor deaths among BDF_1 mice, over 99% occurred between Days 7 and 14 after inoculation. A somewhat "tighter" distribution was obtained for the 7598 "normal" tumor deaths among CDF_1 mice, and over 99% of these deaths occurred between Days 7 and 12.

Based on these results, a reasonable "quality-control" strategy for L1210 control groups has been developed. For BDF_1 mice, any animal dying before Day 7 or after Day 14 is said to be "out of control." In a BDF_1 control group of ten to 21 animals, *up to two* out-of-control mice are allowable, but *three* such mice are allowable in a control group of 22-40 animals, inclusive. Otherwise the entire control group

TABLE 5.—Control-animal survival-time groups

Group	BDF_1 mice			CDF_1 mice		
	Day of death (range)	No. of animals	%	Day of death (range)	No. of animals	%
"Early" deaths	2-6	171	0.43	4-6	17	0.22
"Normal" tumor deaths	7-16	39,360	99.44	7-13	7598	99.61
"Late" deaths	17-26	44	0.11	14-18	13	0.17
"No takes"	>29	5	0.01	19+	0	0

TABLE 6.—Control-group mean and median limits

No. of animals in control group	Control limits (days) for—			
	Mean survival time		Median survival time	
	BDF_1 mice	CDF_1 mice	BDF_1 mice	CDF_1 mice
15	8.9-10.4	8.5-9.8	8.8-10.6	8.4-9.9
20	9.0-10.3	8.6-9.7	8.9-10.5	8.5-9.8
25	9.1-10.3	8.6-9.6	8.9-10.4	8.5-9.8
30	9.1-10.2	8.7-9.6	9.0-10.3	8.6-9.7

and its associated test groups are regarded as being unreliable, and the tests should be repeated. For CDF_1 mice, the same quality-control strategy is recommended, except that an individual animal is said to be out of control if it dies before Day 7 or after Day 12.

This quality-control strategy is extremely conservative; the probability of false rejection of a control group is well under 2.0%. In addition, it offers excellent protection against the inclusion of "early" or "late" deaths in an acceptable control group. The proportion of early deaths in excess of the 7-day lower limit is less than 10% for BDF_1 and less than 5% for CDF_1 mice, and the proportions of late deaths less than the 14- or 12-day upper limits are less than 0.2% and 0.1%, respectively.

Based on the distributions of "normal" mouse survival times, it is also possible to develop a quality-control strategy that controls the mean or the median of the entire control group. Such a strategy is necessarily a function of the control-group size, and recommended control limits for the means and medians of control groups of various sizes are shown in table 6. The control limits shown in this table are 98% limits, ie, the probability of rejecting a control group that is actually "in control" is less than 2%.

LIST OF COMPOUNDS

NSC-195458: 2-propenoic acid, 3-thiocyanato-

NSC-210924MA00: phosphorus (1+), trichloro[[2,6-diamino-5-(3,4-dichlorophenyl)-4-pyrimidinyl]methyl]-, bromide, (T-4)-

NSC-211519: L-glutamic acid, *N*-[4-[[(2,4-diamino-6-pteridinyl)amino]methyl]benzoyl]-

NSC-218441MA00: quinolinium, 6-amino-4-[[3-amino-4-[[[4-[(1-methylpyridinium-4-yl)amino]phenyl]amino]carbonyl]phenyl]amino]-1-methyl-, bis(methyl sulfate)

NSC-218476MA00: β-D-glucopyranose, 6-thio-, 6-(dimethylarsinite)

NSC-D214541MA00: CAS reg. No. 1522-96-9; benzene, 1,1'-[(2-methylene-1,3-propanediyl)bis(oxy)]bis[4-chloro-

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Modified Protocol for the Testing of New Synthetics in the L1210 Lymphoid Leukemia Murine Model in the DR&D Program, DCT, NCI[1]

R. I. Geran, N. H. Greenberg, M. M. Macdonald, and B. J. Abbott[2,3]

The purpose of this now-modified protocol (appendix A) is to select synthetics with possible antitumor activity by using an initial three-mouse prescreen (stage I, step 1) to provide in vivo evaluation of as many compounds as possible, even those received in a small quantity, and by continuing the testing of any initially active drug using more mice per test and using additional regimens.

The reduction in T/C value from 125% to 120% for rating activity will provide for capturing additional active compounds in the prescreen with the knowledge that the number of false positives will increase with the reduced %T/C. These false positives will be eliminated at later screening stages. Any synthetic showing activity in the original three-mouse assay is to be retested in the originally active regimen and is to be tested by as many of the other three regimens as possible with available supply. If the supply is not sufficient for all of this testing, retesting in the originally active regimen takes precedence and a refill is not to be requested unless activity is seen in two separate experiments.

Appendix B is an outline of the general plan for stage I testing of a synthetic and appendix C is a list of the activity criteria for the various stages of testing.

The screening laboratory is to attempt to achieve maximum utilization of each compound, exercising judgment and modifying doses as experience with a compound accumulates. These comments pertaining to the current protocol are offered merely as guidelines.

A. Unless otherwise instructed, September 1972 protocols[4] still apply.

   Examples:

   1. When toxicity data are supplied, initial doses should be based on that information. Protocol 4.101
   2. For a multidose assay to be considered "inactive and complete" there must be at least two nontoxic inactive dose levels. Protocol 12.100

B. In stages I and II, a dose of 600 mg/kg/injection is not to be exceeded.

C. "Confirmed activity" is the required %T/C in two separate experiments versus the same five-digit test system by the same drug route and drug schedule, either at the original laboratory or at another laboratory.

D. "Reproduced activity" is the required %T/C in two separate experiments versus the same five-digit test system not by the same drug route and schedule, either at the original laboratory or at another laboratory.

E. If supply does not permit all testing, confirmation of previous activity takes precedence.

F. Rule-of-thumb factors to use in adjusting dose levels to new regimens are: one single = four intermittent = eight chronic (ie, 1S = 4I = 8C). The following is an example of this relationship.

Regimen	Top dose (mg/kg/injection)
Single (S)	400
Intermittent (I)	100
Chronic (C)	50

In no case is a 600-mg/kg/injection dose to be exceeded.

G. If a material is active, the animal weight change difference [AWCD (T-C)] is ignored, but a negative %T/C with AWCD (T-C) ≥ 4 g is considered toxic.

H. If any synthetic has reproduced activity (T/C ≥ 120%), all stage II regimens are to be tested that were not tested in stage I. If a supply is not available for all regimens, the screener is to request a refill through the screening services contractor.

I. In vivo screening is now to be scheduled for all compounds of which at least 15 mg is received.

J. No material is to be transshipped by the original laboratory for in vivo confirmation except on specific request.

K. Synthetics received in quantities of 179 mg or less that are negative in stage I, step 1 should be routinely transshipped by the in vivo screening laboratory for KB testing if the supply permits. No other synthetic is to be transshipped for KB testing unless transshipment is specifically requested.

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[1]Drug Research and Development Program (DR&DP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI).

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[3]*Reprint requests to:* Dr. R. I. Geran, Drug Evaluation Branch, DR&DP, DCT, NCI, 8300 Colesville Rd, Blair Bldg, Rm 517, Silver Spring, Md 20910.

[4]*Cancer Chemother Rep* Part 3, vol 3(2):1-102, 1972.

APPENDIX A

TABLE 1.—Regimens and dose levels for stage I, step 1 testing

Amount available (mg)	Regimen	Doses (mg/kg/injection)	Mg needed for step 1
≥ 810	qd, Days 1-9	200, 100, 50	237
540-809	q4d, Days 1, 5,9	400, 200, 100	158
360-539	q4d Days 1,5	400, 200, 100	105
180-359	Day 1 only	400, 200, 100	53
90-179	Day 1 only	400	30
45-89	Day 1 only	200	15
15-44	Day 1 only	200	15

Any material active in the three-mouse assay (stage I, step 1) but inactive in the first repeat six-mouse assay (same regimen) is considered complete unless quantity permits testing by other schedules. No request for a refill is to be made for additional schedules.

If a material is active in the three-mouse assay and the quantity is not sufficient (QNS) to repeat with six mice the regimen in which activity was found, a refill should be requested.

If a material shows reproduced activity in stage I, step 2 and quantity is insufficient for all stage II testing, a refill should be requested.

Any material in insufficient quantity for stage III testing will be reported QNS by the Drug Evaluation Branch staff, who will have responsibility for scheduling this testing.

Stage I Screen for Synthetics

For all of the steps in stage I screen for synthetics, use—
Tumor: L1210 implanted ip (3LE21)
Drug route: ip
Parameter: mean survival time
Criterion of activity: T/C ≥ 120%

Stage I, step 1 (initial testing)
No. of mice/test: three
No. of regimens: one
No. of doses: three (when quantity permits)
Dose levels and regimen: see table 1
Followup: if supply is sufficient, go to stage I, step 2a; if supply is insufficient for stage I, step 2a, go to stage I, step 2b

Stage I, step 2a (repeat of stage I, step 1 with adequate supply)
No. of mice/test: six
No. of regimens: from two to four
No. of doses: four—2x, x, 0.5x, and 0.25x where x = optimal dose from stage I, step 1; an exception is when original material received is ≤ 179 mg (see comment F regarding regimen factor)
Regimens: the same as stage I, step 1 plus any or all of the others listed in table 1 permissible with available supply

Stage I, step 2b (repeat of stage I, step 1 with limited supply)
No. of mice/test: six
No. of regimens: one
No. of doses: four—2x, x, 0.5x, and 0.25x where x = optimal dose in stage I, step 1
Regimen: same as stage I, step 1
Criterion for passing stage I (material classification code "OS"): a T/C ≥ 120% in a three-mouse test (any regimen) plus a T/C ≥ 120% in a six-mouse test (any regimen)

Stage II Screen for Synthetics

Test system: L1210 implanted ip (3LE21)
No. of mice/test: six
Drug route: ip
No. of regimens: four
No. of doses: five-2/3 intervals surrounding x where x = most effective dose from stage I (see comment F regarding regimen factor)
Regimens: single, Day 1; q4d, Days 1 and 5; q4d, Days 1, 5, and 9; and qd, Days 1-9. All regimens not tested in stage I *and* all regimens active at least once in stage I, must be tested in stage II.
Criteria for passing stage II: (a) any one T/C ≥ 200%, or
(*b*) an average T/C ≥ 125% from the optimal responses (any regimen) in each of two six-mouse experiments with each T/C value used to calculate the average ≥ 120%.

Stage III Screen for Synthetics and Natural Product Isolates

INPUT

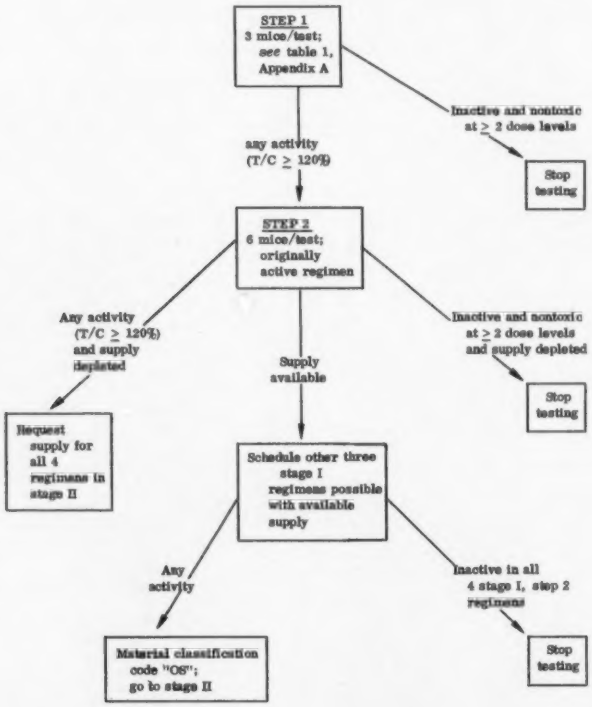
Analog

1. Determine interest in type
2. Testing directed specifically to type; based on biologic, biochemical, pharmacologic, physical-chemical properties of parent

Unique

Type I schedule dependency (including oral and sc treatment, and toxicity in normal mice)
Early 3PS31 (ip P388 leukemia)
Early 3B131 (ip B16 melanoma)
Early 3B132 (sc B16 melanoma)
Advanced 3LE32 (sc L1210 leukemia)
Advanced 3PS32 (sc P388 leukemia)
Early 3LL32 (sc Lewis lung carcinoma) and, if active, advanced 3LL32
Early 3LE37 (ic L1210 leukemia), 3PS37 (ic P388 leukemia), 3B137 (ic B16 melanoma), or 3EM37 (ic ependymoblastoma)
Other secondary screens

APPENDIX B.—General plan for testing a synthetic in stage I


STEP 1
3 mice/test;
see table 1,
Appendix A
Inactive and nontoxic at ≥ 2 dose levels
Stop testing
any activity (T/C ≥ 120%)
STEP 2
6 mice/test;
originally active regimen
Any activity (T/C ≥ 120%) and supply depleted
Supply available
Inactive and nontoxic at ≥ 2 dose levels and supply depleted
Stop testing
Request supply for all 4 regimens in stage II
Schedule other three stage I regimens possible with available supply
Any activity
Inactive in all 4 stage I, step 2 regimens
Material classification code "OS"; go to stage II
Stop testing

No dose is to exceed 600 mg/kg/injection.
Toxic tests must be repeated at lower doses.

APPENDIX C —List of activity criteria for synthetics

Criterion of activity in stage I testing

	%ILS
L1210 leukemia	20

Stage II testing is started by the screening laboratory when a material meets the requirement for a material classification code of "OS" (see page 16).

Criterion of activity in stages II or III testing—one of the following:

	%ILS		%ILS
L1210 leukemia	25 (average)	B16 melanoma	40
P388 leukemia	25	Lewis lung carcinoma	40

Criterion of activity required for candidate for DN-2—one of the following:

	Confirmed %ILS		Confirmed %ILS
L1210 leukemia	50	B16 melanoma	50
P388 leukemia	75	Lewis lung carcinoma	50

Preclinical Characterization of Candidate Antitumor Drugs

Abraham Goldin, Randall K. Johnson, and John M. Venditti[1,2]

Contents

In vivo pharmacologic testing
A. Drug toxicity in normal mice
B. Drug toxicity in mice with tumors
C. Pharmacologic determination of effective drug levels, for comparison with direct measurement of drug levels
Drug activity in a spectrum of tumor systems
In vivo antitumor testing of drugs
A. Drug effect on tumor growth
B. Drug effect on survival time of tumorous host; schedule and route of administration
C. Relation of dose-response curves in treated tumor-bearing mice to dose-response curves in normal mice
D. Drug effect on tumor cell kill and on cure of animals
E. Intracranial tumor and tumor at other sequestered sites
F. Resistance
G. Metastasis
H. Drug action on immune response of host
1. Effect of drugs on cell-mediated immunity
2. Effect of drugs on host-versus-graft reactions
3. Effect of drugs on graft-versus-host (GVH) reactions
4. Effect of drugs on lymphocyte proliferative response in vitro
5. Effect of drugs on humoral immune responses
I. Combination chemotherapy
J. Combined modalities
1. Surgery plus chemotherapy
2. Immunity plus chemotherapy
3. Radiation plus chemotherapy
K. Special test models
L. Carcinogenic activity
M. Chronobiologic influences on drug activity
In vitro characterization of drugs
Application of methodology to selection of analogs
Conclusion

Following detection of activity for a new compound in a primary antitumor screening program the major objective of the preclinical program is to characterize the activity of the compound with respect to parameters that have relevance for the clinic. Although once definitive activity has been confirmed for a compound it could be considered a candidate for clinical development with little or no further preclinical characterization, the limited information available under such circumstances would clearly represent too severe a handicap with respect to successful drug choice or usage. It would appear that a reasonable thesis entails the gathering of comprehensive information about a new compound for decision making in drug development. We feel that it is possible to extend the preclinical evaluation, in accordance with planned protocols, well beyond the procedures currently employed routinely in chemotherapy screening programs. This could be accomplished readily since there is a broad base of well-defined preclinical models from which to choose specific types of testing to provide data of greatest pertinence to the clinic. There are sophisticated laboratories conducting preclinical evaluation which could readily accomplish expanded tests employing standardized models in a relatively short time following the initial identification and confirmation of activity.

Currently, at the National Cancer Institute (NCI), once a compound has been determined to be active in a primary antitumor screen, it is subjected to a preliminary schedule-dependency evaluation (table 1) (1,2) and is tested in several tumor systems. In accordance with the established protocols of the NCI, if the new compound is a synthetic agent determined to be active in leukemia L1210, it is then tested in leukemia P388, B16 melanoma, and Lewis lung tumor. Natural products determined to be active in leukemia P388 are then subjected to testing in leukemia L1210, B16 melanoma, and Lewis lung tumor. In certain cases, such as with analogs of known antitumor agents, additional tumor test systems may be employed, and the compound may be subjected to in vitro characterization. Over a period of time the drug may be tested in a variety of systems, but this is generally not accomplished in a systematic manner on a broad scale. Also, the testing may not be conducted within any specific time frame relative to the preclinical toxicologic development of a potentially active drug of interest.

The following represents an outline of areas of interest for which, based on published procedures, additional definitive protocols could be drawn up in a comprehensive drug development program, such as the one reported for the chemotherapy program at the NCI (3). It would entail consideration for movement of procedures from concurrent and supplemen-

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TABLE 1.—Protocol for initial (Type I) schedule-dependency study against L1210*† [*from* ref 1]

Drug route	Treatment schedule‡
Ip,sc,oral	Once on Day 1 only
Ip	Every 3 hours on Day 1 only
Ip	Once daily, Days 1-5
Ip,sc,oral	Once daily, Days 1-9
Ip	Once every 4th day, Days 1,5,9
Ip	Every 3 hours, every 4th day, Days 1,5,9
Ip	Once every 8th day, Days 1 and 9
Ip	Every 3 hours, every 8th day, Days 1 and 9

*The experimental method for the Type I L1210 schedule-dependency test is generally that used for primary screening modified as follows. Each treatment schedule includes 4 or 5 dose levels, ranging from an ineffective or minimum effective dose to a frankly lethal dose, given to parallel groups of 8-10 leukemic mice and 6-8 normal mice. For each schedule, as the dose is increased, the survival time of leukemic mice is increased until host toxicity becomes limiting. A further increase diminishes survival time. Relative effectiveness among schedules is based on the maximum increase in lifespan of leukemic mice over controls provided by the optimum dose or the highest non-lethal dose.

†In some studies at the Southern Research Institute a similar but modified protocol uses treatment on Day 2 only (single injection and q3h), intermittent treatment on Days 2, 6, 10, and 14 (one treatment each day and q3h on each day), and daily treatment on Days 2-16. Results for drugs examined by both protocols have not differed significantly.

‡Day 0 = day of ip leukemic implant (10^5 cells/mouse).

tary array of test systems (3,4) into the direct linear array for the development of a new drug. In 1962 "A Manual on Quantitative Drug Evaluation in Experimental Tumor Systems" was published (5,6) which called for "a compilation of quantitative experimental therapeutic data which when expanded might be employed (*a*) as a base line for comparing the 'activity' of new agents under consideration for clinical trial, (*b*) in the selection of additional tumor systems with the view to better 'coverage' in primary screening and specific systems for study and ranking of congeners, (*c*) as a basis for long-term planning and analysis of parallelisms in drug response of specific experimental systems and specific types of human clinical cancer, and (*d*) as a guide to chemists concerned with structure-activity relationships and new drug design." This current review was prepared with a view to the furthering of these and related objectives.

Most of the examples cited in this review are taken from extensive experience with the well-characterized leukemia L1210 system. It is not suggested that further extension of secondary drug evaluation be limited to this one system. However, the techniques and principles evolved from the study of leukemia L1210 should be applicable to other experimental tumor systems including both rapidly proliferating hematologic malignancies and slow-growing solid tumors.

IN VIVO PHARMACOLOGIC TESTING

A. Drug Toxicity in Normal Mice

Following identification of activity in a primary screening system, a drug can be subjected at a relatively early stage of development to in vivo pharmacologic testing. These experiments could serve as indications of schedules which might be used in therapeutic studies and could allow the selection of therapeutic dose levels relative to host toxicity. In a recent report (7) it was concluded that the determination of a safe starting dose for clinical phase I studies should take into account toxicology data in normal and tumor-bearing mice as well as dog and monkey data (8). The lethal doses for 10% and 90% of normal mice and the optimal dose in L1210-bearing mice afforded good quantitative prediction of human toxicity. Complete dose-mortality curves can be obtained in normal mice employing a series of schedules of drug administration including single treatment and treatment for varying total intervals including continuous treatment. For each total interval of treatment the number of treatments may be varied. Also, for each day of treatment a range of total numbers of treatments could be employed. In addition, varying types of intermittent schedules could be tested such as daily or intermittent schedules on alternate weeks, as well as novel types of schedules.

Observations can be made during these studies of any acute or chronic toxic effects. The rapidity of occurrence of toxicity may be determined, as well as the reversibility of toxicity and rate of host recovery once treatment has been discontinued. For each of the schedules tested, comparison can also be made of the individual dosage and total dosage for equivalent toxic response as well as of alteration in dose-response slope.

Comparison may be made of the influence of routes of administration, including subcutaneous (sc), intraperitoneal (ip), intravenous (iv), and oral with and without fasting.

A typical dose-response curve is illustrated in figure 1 (5). It represents the mortality response over a series of dosages of cyclophosphamide in the Holtzman rat. Drug administration was ip, daily for 5 days, and the animals were observed for mortality for 21 days. In this study each point represents ten

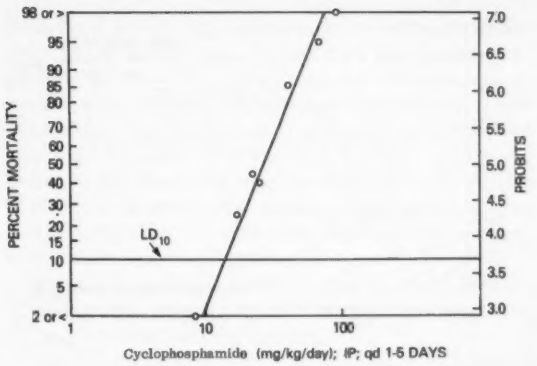


FIGURE 1.—Mortality response to cyclophosphamide in the Holtzman rat. The drug was administered ip daily for 5 days and the animals were observed for mortality for 21 days. Each point represents 10-20 animals [*from* ref 5].

to 20 animals. From the data, the LD50, LD10, and slope of the dose-mortality curve can be calculated. Comparison can then be made of the dose-response curves, including these parameters, for the various schedules of treatment tested.

Dose-response curves for the toxicity of actinomycin D on a number of schedules of administration in BDF_1 mice are illustrated in figure 2 (9) and the individual and total dose LD10 as well as the total dose relative to a single dose for each schedule are summarized in table 2 (9). It is clear that there is very little difference in the slopes of the dose-response curves for the various schedules of therapy with this drug. Also, the total dose LD10 is equal to or lower on the various schedules than that observed with single treatment, indicating that toxicity is essentially cumulative and that there is little or no recovery from host toxicity between treatments.

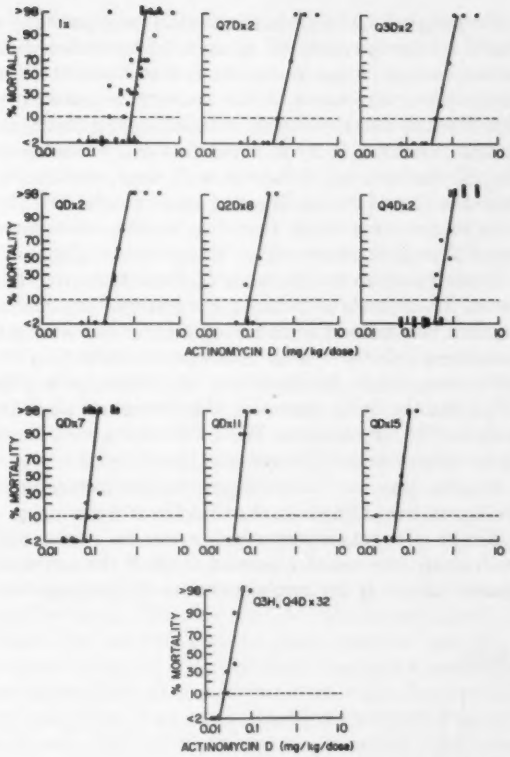


FIGURE 2.—Toxicity of actinomycin D on several schedules of administration in normal BDF_1 mice. Percent mortality is plotted in a probit transformation on the ordinate. The dose of actinomycin D in mg/kg/injection is plotted logarithmically on the abscissa. Each point represents a group of 6-10 mice treated at a given dosage level. Regression lines are drawn through the points. The dashed line represents the LD10 level [*from* ref 9].

TABLE 2.—Single-dose and total-dose LD10 levels for actinomycin D in BDF_1 mice [*from* ref 9]

Schedule	LD10 (mg/kg)*		
	Per dose*	Total dose	Total dose relative to single dose
One dose	0.87	0.87	1.0
qd × 2	0.29	0.58	0.7
q3d × 2	0.47	0.94	1.1
q4d × 2	0.47	0.94	1.1
q7d × 2	0.45	0.90	1.0
qd × 7	0.080	0.56	0.6
qd × 11	0.050	0.55	0.6
qd × 15	0.054	0.81	0.9
q2d × 8	0.11	0.88	1.0
q3h × 8, q4d × 4	0.026	0.83	1.0

*Calculated from regression lines.

Investigations in normal animals may provide a basis for comparison of structurally unrelated or related active drugs. In one study (figs 3 and 4) (10) a comparison was made of the toxicity in mice of 5-fluorouracil and the closely related analog ftorafur. It was observed that on repetitive daily treatment (fig 3) toxicity of 5-fluorouracil was cumulative whereas ftorafur toxicity was less cumulative. The rate of recovery from ftorafur toxicity was more rapid than that observed for 5-fluorouracil (fig 4).

Determination can be made of the influence of the sex of the animals on toxicity. An example in which a marked sex difference in toxicity occurred is in the treatment of mice with aminopterin (fig 5) (11). Following single ip injections of aminopterin into CF_1 mice the drug was more than twice as toxic for male as for female mice. The LD50 was about 2 mg/kg in male mice and 4.5 mg/kg in female mice.

Studies may also be conducted on the influence of the age of the animals on the toxicity of a new drug.

Means of controlling toxicity can be evaluated, such as by the use of a second drug. If the antineoplastic agent is an antimetabolite the metabolite-antimetabolite relationships can be investigated (12).

The nature of the toxicity can be defined for the various schedules (13,14). This involves determination of the hematologic response and myelosuppressive activity of the drug for the various schedules at

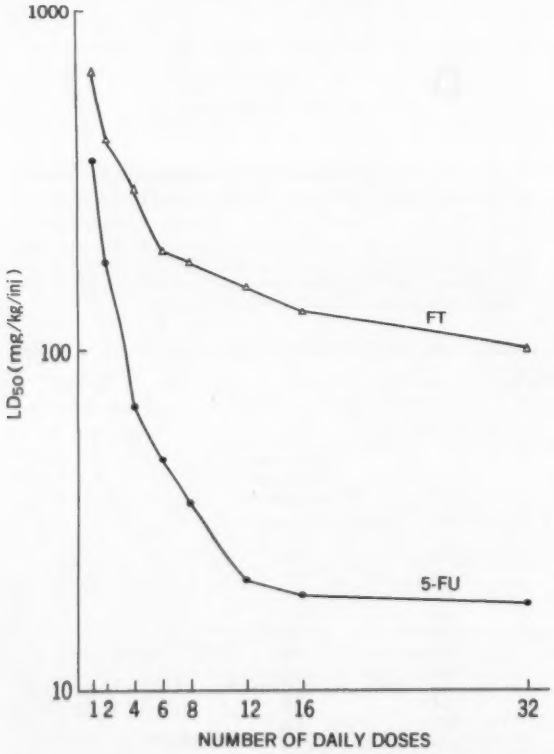


FIGURE 3.—Cumulative toxicity of 5-fluorouracil and ftorafur (FT) in mice [*from* ref 10].

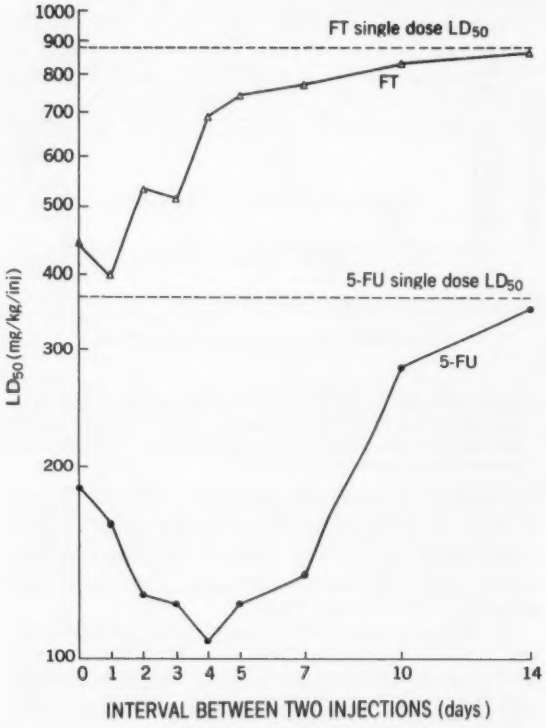


FIGURE 4.—Recovery of mice from toxicity of 5-fluorouracil and ftorafur (FT) [*from* ref 10].

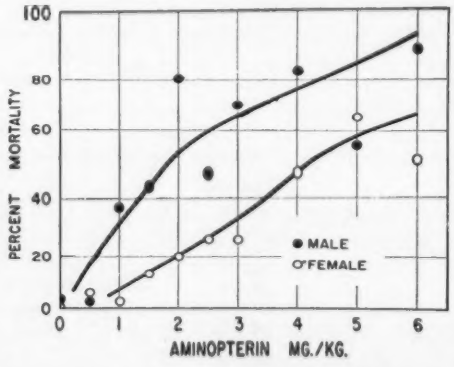


FIGURE 5.—Sex difference in toxicity to aminopterin in adult CF_1 mice. Single ip injection. Mortality at 10 days after treatment [*from* ref 11].

both toxic and tolerated doses. It includes the kinetics of suppression and recovery of the elements of peripheral blood including total and differential leukocyte, erythrocyte, platelet, and hemoglobin determinations. The occurrence of any leukemoid reaction may also be noted. The kinetics of bone marrow depression and recovery may be determined by means of direct marrow counts, spleen colony assay, and so forth. Alteration of standard biochemical parameters should also be determined.

Histologic and biochemical studies could be conducted on the kinetics of cell damage and repair of the gastrointestinal epithelium.

Specific organ toxicity or selective organ accumulation may be investigated.

Pharmacodynamic characterization may include physiologic distribution, duration of blood levels, biochemical transformation, activation of liver microsomal enzymes, excretion, drug uptake by specific cellular elements, specific incorporation into DNA, and so forth.

The responses of the immune system to the drug may be determined, including any immunosuppressant action or immunoenhancement and determination of extent and duration as will be discussed below.

Overall, toxicologic studies in mice may provide information for optimizing dosages and schedules based on the kinetics of parameters of damage and recovery.

The toxicity of folic acid antagonists may be influenced markedly by the schedule of administration. A treatment involving two doses of aminopterin spaced 1 day apart was considerably more toxic to mice than a single treatment. It was not until the two doses were spaced 4 days apart that sufficient recovery of the host occurred so that the two doses resulted in only additive toxicity (12).

On the basis of both animal and clinical investigations Karrer et al (15) concluded that postoperative human bronchial carcinoma and transplantable murine leukemia L1210 could best be treated with a dosage of cyclophosphamide that produced mild leukopenia of between 2 and 3×10^3 leukocytes/mm^3 of blood in long-term adjuvant therapy.

Drug treatment during the log phase of bone marrow repair may be expected to result in greater toxicity than when the drug is given after the repair has been completed or during any overshoot period. DeWys et al (16) investigated hematopoietic recovery after large doses of cyclophosphamide and correlated the proliferative state with sensitivity. They observed that hematologic recovery following large doses of cyclophosphamide was characterized by early rapid recovery of marrow stem cells followed successively by recovery of circulating stem cells, total marrow cellularity, and circulating blood cells. It was determined that if the interval between the first and second treatment with large doses of cyclophosphamide was too brief, greater hematopoietic toxicity occurred. It was not necessary to wait for complete hematopoietic recovery before giving a second treatment, but it was found that treatment should be delayed until the stem cell population had recovered and was no longer in an active proliferative state. In the study it was found that this coincided with the earliest stages of recovery of the circulating blood cells. Also, it was observed that on the fifth day following an initial treatment the stem cell population was less sensitive to cyclophosphamide than the stem cell population in previously untreated marrow. Animals with this less sensitive stem cell population were capable of surviving a larger dose of cyclophosphamide compared to previously untreated animals.

In another study, Young et al (17) reported that following a massive single treatment with cytosine arabinoside (1000 mg/kg) toxicity was increased when a second dose was given 12-36 hours following the first dose. This time period coincides with that at which the normal tissues, bone marrow and gastrointestinal tract, are synthesizing and apparently repairing DNA at an above-normal rate. On the other hand, there was reduced toxicity when the second dose was delayed 96-120 hours, at which time bone marrow and gastrointestinal tract were synthesizing DNA at a lower rate.

Where desirable or where gross examination indicates possible tissue or organ damage, the pathology resulting from treatment with a drug may be determined in mice for various schedules of treatment. These observations may in turn be of interest for comparison with observations in larger animals. Observations can be made of the extent of local tissue reaction, either at the sc or iv site or after ip or oral administration. Systemic pathologic effects can be determined at autopsy. Gross examination may be made of the lung, spleen, gastrointestinal tract, liver, kidney, endocrine organs, and lymphoid tissues. The tissues may be sectioned and histologic examination conducted of heart, lung, spleen, gastrointestinal tract, pancreas, liver, kidney, adrenal, testis, thymus, lymph nodes, bone marrow, brain, thyroid, and skeletal muscle.

B. Drug Toxicity in Mice With Tumors

The toxicity studies in tumor-bearing animals may be modeled after the studies outlined above for normal animals. Comparative toxicity studies may be conducted to determine the extent of reduced drug tolerance in the tumorous animal and the influence

of factors on the toxic response such as the stage of development of the disease, the localization of the disease, and so forth. Tolerated schedules of a drug may be different in the tumorous animal as well as the various toxic parameters including hematologic response and myelosuppressive activity, damage to gastrointestinal epithelium, specific organ toxicity, and pharmacodynamic and biochemical characteristics.

C. Pharmacologic Determination of Effective Drug Level, for Comparison With Direct Measurement of Drug Levels

Although there has been considerable emphasis on the development of biochemical methods for the determination of the extent and duration of blood and tissue levels of a new drug and of any active transformation products, the relationship of these findings to interpretation of the antitumor properties of the drug frequently presents difficulties. Quantitative biologic procedures can be employed for the determination of the effective drug levels of chemotherapeutic agents and such studies may supplement the biochemical findings. Drugs such as cyclophosphamide and the nitrosoureas which result in a marked antitumor effect when administered as a single injection lend themselves well to study by the biologic procedures. By injection of the drug at various intervals prior to the inoculation of the tumor cells and by comparison of the antitumor effect with that observed when the drug and tumor cells are inoculated concomitantly, it is possible to estimate the duration of effective drug levels. These studies are particularly pertinent for drugs such as cyclophosphamide which require activation by transformation prior to being effective. In one study (fig 6) (18) it was observed that relative to treatment at the same time as sc leukemic inoculation there was a sharp drop in therapeutic effectiveness when melphalan was administered 1 hour prior to inoculation of the leukemic cells. In a more extensive report, for which the methodology is described in detail (19), studies of effective drug levels were conducted with cyclophosphamide and CCNU. In this procedure a standard dose-response curve is established in which the test drug is administered concomitantly with the inoculation of a designated number of leukemia L1210 cells. The parameters of response include the percentage of cures (60-day survivors) and the median survival time (MST) of the animals. The test drug is also administered at a series of time intervals prior to the inoculation of leukemic cells and the dose level equivalence at the time of leukemic inoculation is estimated by comparing the observed therapeutic response with the results obtained with the standard curve for concomitant treatment. For both cyclophosphamide and CCNU, curves were established relating the percent retention of administered drug dose to the elapsed time between drug administration and subsequent leukemic inoculation. In the case of cyclophosphamide the apparent retained levels of administered drug decreased rapidly and the active moiety was estimated to have a half-life in

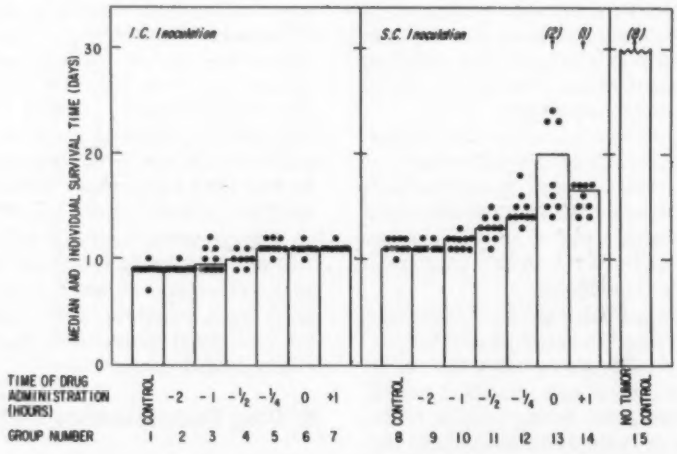


FIGURE 6.—Duration of effectiveness of melphalan in mice given ic or sc inoculations of 0.05 ml of a 1/100 dilution of leukemic whole blood; 1 sc injection of melphalan (15 mg/kg) given before (-), simultaneously with (0), or after (+) inoculation; tumor-free controls were normal mice given injections of drug; numbers in parentheses = mice living > 30 days; 8 mice each in groups 4, 6, 7, and 15, and 10 mice in all other groups [*from* ref 18].

the host of 17 minutes. The curve for percentage retention of administered CCNU had a more shallow slope and the half-life of the active moiety in the host was estimated to be 94 minutes. The experimental design for cyclophosphamide and the "decay" curves for cyclophosphamide and CCNU are presented in table 3 and figure 7. The results of such studies provide a basis for comparison with concentration-time (C × T) relationships for blood and tissue levels by direct methods of measurement and may help to more closely relate the biochemical observations to the therapeutic observations.

Employing this methodology there are areas that are worthy of further exploration. The influence of multiple pretreatment schedules on retention of effective blood levels may be explored. The influence of pretreatment on posttreatment effectiveness (extent of additivity of effect) may be investigated. The influence of drug A on the duration of effective levels of drug B (two sequential pretreatments) could be determined. Drug A could be another antitumor agent or another type of drug including those generally employed in the overall treatment of the cancer patient. The influence of temporal relationships of administration of a metabolite with an antimetabolite on the duration of effective levels of the antimetabolite may also be suitable objects for investigation.

Although the system as employed appears to be most suitable for estimation of effective levels of drugs that have definite antitumor effectiveness as a single injection, it would appear possible to extend this type of methodology to drugs that are less cytotoxic on single-dose treatment but more effective on multiple-dose administration.

DRUG ACTIVITY IN A SPECTRUM OF TUMOR SYSTEMS

Although currently a new drug found to be active in the NCI screening program against L1210 or P388 leukemia is also tested against B16 melanoma and the Lewis lung tumor, it would be worthwhile to de-

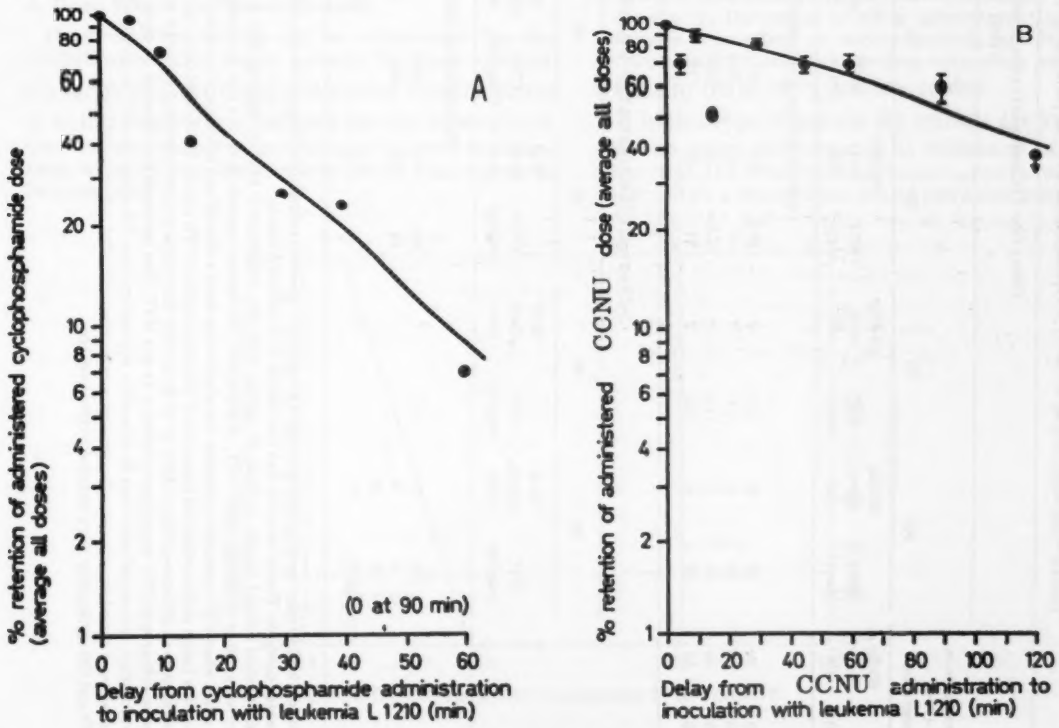


FIGURE 7.—*A.* Each individual point represents the average retention of cyclophosphamide expressed as a percentage of the doses actually administered. The antileukemic half-life of 17 mins was estimated from the straight line drawn by inspection [*from* ref 19]. *B.* Each individual point represents the average apparent retention of CCNU expressed as a percentage of the doses actually administered. The antileukemic half-life of 94 mins was estimated from the straight line drawn by inspection [*from* ref 19].

TABLE 3.—Duration of drug levels of cyclophosphamide (CYC) as indicated by residual antileukemic efficacy* [*from* ref 19]

CYC (single dose, mg/kg)	Simultaneous CYC† (controls)		Delay from CYC administration to inoculation with leukemia L1210 (mins)													
			90		60		45		30		15		10		5	
	MST (days)	60-Day surv. (%)	MST (days)	60-Day surv. (%)	MST (days)	60-Day surv. (%)	MST (days)	60-Day surv. (%)	MST (days)	60-Day surv. (%)	MST (days)	60-Day surv. (%)	MST (days)	60-Day surv. (%)	MST (days)	60-Day surv. (%)
			Observed antileukemic effect of administered CYC													
357	>60	94	10	0	13	0	>58.5	50	>37.5	50	>60	75	>60	75	>60	75
250	>60	88	10	0	13	0	19	12	23.5	25	16.5	0	>60	100	>60	62
175	>60	88	10	0	11.5	0	15.5	0	15.5	12	>60	62	17.5	25	>60	88
123	>60	81	10	0	10	0	11	0	13	0	16.5	25	>60	62	>60	75

CYC (mg/kg)	Delay from CYC administration to inoculation with leukemia L1210 (mins)													
	90		60		45		30		15		10		5	
	CYC (mg/kg)	% of admin. dose	CYC (mg/kg)	% of admin. dose	CYC (mg/kg)	% of admin. dose	CYC (mg/kg)	% of admin. dose	CYC (mg/kg)	% of admin. dose	CYC (mg/kg)	% of admin. dose	CYC (mg/kg)	% of admin. dose
	Estimated retention of CYC‡													
250§	0	0	17	7	52	21	60	24	60	24	250	100	88	35
175	0	0			48	27	48	27	88	50	78	44	200	100
123	0	0							60	49	88	72	110	89
Average %	0		7		24		26		41		72		95¶	

*16 BDF_1 male mice (19-27 g) were used in each treatment group in the treated controls (simultaneous drug administration) and 8 mice in all other treatment groups; 16 animals were employed for untreated controls. Tumor (ascites) was inoculated sc; treatment was sc.

†Additional simultaneous control results include: 86 mg/kg, MST>60, survivors = 56%; 60 mg/kg, MST = 16.5, survivors = 25%; 42 mg/kg, MST = 15, no survivors; 29 mg/kg, MST = 14, no survivors; 20 or 14 mg/kg, MST = 13, no survivors; untreated controls, MST = 11.5, no survivors.

‡Each observed effect when CYC treatment precedes inoculation has been referred back to the control data in order to determine the amount of simultaneous CYC yielding the same outcome. This amount is taken as the estimated retention of the administered CYC and can be expressed as a percentage of the dose actually administered. For example, the dose of 250 mg/kg administered 60 mins in advance yields an MST of 13 days, while this same outcome is yielded by either 14 or 20 mg/kg of simultaneous CYC. From smooth curves it was estimated that 17 mg/kg of simultaneous CYC would have yielded a 13-day MST. The estimated retention of 17 mg/kg is 7% of the actually administered dose of 250 mg/kg. If the estimated retention is a true situation, this would mean that 60 mins after administration, 7% as much is still available to the host as was available initially.

§357 mg/kg which showed some lethal toxicity for some groups was not included in the calculations.

¶The data for 175 and 123 mg/kg only were included in this average.

termine the activity of the drug in a broader spectrum of well-characterized tumor systems. The establishment of such a spectrum of antitumor activity for a new agent, as employed earlier by Schmidt (20) and Larionov (21) and as practiced for a period in the program at the NCI (22,23), can be readily accomplished and may be most useful for comparison with the spectra of activity of the known antitumor agents. It may be an important contribution in the evaluation of the potential usefulness of congeners. A definitive increase in the effectiveness of a new congener in a tumor system relatively resistant to the parent compound already in clinical use would serve as an important index for focusing attention on the congener. The activity of representative established antitumor agents in a number of tumor systems is illustrated in table 4 (9), and a classification of the activity of actinomycin D in a spectrum of tumor systems is shown in table 5 (9).

IN VIVO ANTITUMOR TESTING OF DRUGS

A. Drug Effect on Tumor Growth

Dose-response curves can be established for the inhibition of solid tumor growth. In these systems the tumor cells are usually implanted into the mouse sc or intramuscularly (im) and the size or weight of the tumor is measured periodically in order to determine whether significant inhibition of tumor growth has occurred.

In one system the maximum tolerated dose for the drug (usually taken as the dose giving a 10% mortality response, LD10) is determined in normal animals and the extent of inhibition of tumor growth (maximum effectiveness) is measured for that dose. This is illustrated in a study by Skipper and Schmidt (fig 8) (5). The ratio of the maximum tolerated dose (LD10) to the minimum effective dose (lowest dose resulting in a specified antitumor effect), the "therapeutic index," is a measure of the margin of safety with which a drug can be employed. The higher the ratio, the greater the latitude in drug usage. Employing this type of system the relative antitumor effectiveness of drugs can be compared at their respective maximum tolerated doses (LD10) or at some other designated level of drug toxicity for the host. Drug comparisons can also be based on the therapeutic indices of the compounds.

In the determination of the influence of therapy on tumor growth, the schedule of treatment may be important and comparisons can be made of single-treatment and multiple-treatment schedules.

Similarly, the route of drug administration may influence drug effect on tumor growth, and it would be important to conduct testing with drug administration by the sc, iv, ip, and oral routes.

If in this type of system the animals are killed in order to weigh the tumor, it is necessary to determine that the inhibition of tumor growth was not observed at a dose which would have resulted in de-

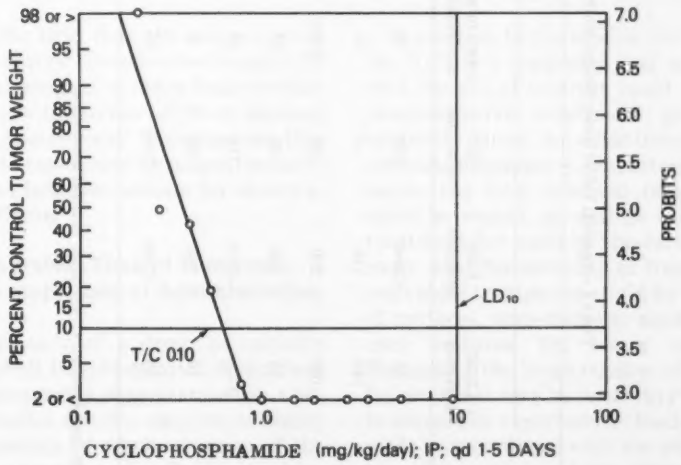


FIGURE 8.—Effect of treatment with cyclophosphamide on Walker carcinosarcoma 256. Average data from 6 experiments are presented with 12-36 animals per point.

$$\text{Therapeutic index} = \frac{\text{LD10}}{\text{T/C 0.10}} = \frac{14}{0.66} = 21$$

Maximum effectiveness = T/C at LD10 = 0 [*from* ref 5].

TABLE 4.—Optimal ILS or tumor weight inhibition (TWI) of seven clinically active antitumor agents [*from* ref 9]

Drug	% ILS									% TWI	
	L1210 leukemia	P388 leukemia	B16 melanoma	Lewis lung carcinoma	P1534 leukemia	P288 leukemia	L5178Y leukemia	Dunning leukemia	Gardner lympho-sarcoma	Walker 256 carcino-sarcoma	Adenocar-cinoma 755
Actinomycin D	45	Cure†	100	10	0	55	26	20	75	65	100
Methotrexate	140	120	0	15	60	190	88	95	200	95	55
Cyclophosphamide	160	200	60	Cure	70	100	*	Cure	200	90	100
5-Fluorouracil	95	120	45	25	*	90	55	65	>120	72	95
BCNU	Cure	Cure	110	45	165	100	160	Cure	200	100	85
Vincristine	40	140	80	0	Cure	80	25	80	200	80	45
Adriamycin	50	Cure	Cure	15	*	*	*	*	*	90	*

*Not tested.
†Greater than 50% long-term survivors after optimal treatment.

TABLE 5.—Activity of actinomycin D in experimental tumor systems* [*from* ref 9]

Tumors which could be cured by actinomycin D treatment	Tumor systems in which actinomycin D markedly increased lifespan or inhibited local tumor growth	Tumors against which actinomycin D had borderline activity	Tumor systems in which actinomycin D was inactive
P388 leukemia	B16 melanotic melanoma (100)	L5178Y lymphatic leukemia (26)	ADJ-PC-20 plasma cell
Ridgway osteogenic sarcoma	L1210 leukemia (45)	P355 leukemia (30)	ADJ-PC-22 plasma cell
	Gardner 6C3HED lymphosarcoma (75)	Lymphoma 8 (rat) (26)	Lieberman plasma cell No. 1 (LPC-1)
	Mecca lymphosarcoma (83)	Murphy-Sturm lymphosarcoma (rat) (78% TWI)	Friend virus leukemia
	P288 lymphocytic leukemia (55)	Walker carcinosarcoma 256 (rat) (65% TWI)	Hepatoma 129
	P1081 chloroleukemia (145)	AK leukemia (0-47, variable)	AK4 lymphoid leukemia
	P329 reticulum cell sarcoma (66)		Lewis lung carcinoma
	Lymphoma 4 (94)		MPC2 plasma cell
	Sarcoma 180 (83% TWI)		Osteogenic sarcoma He 10734
	Adenocarcinoma 755 (100% TWI)		P1534 leukemia
			P1798 lymphosarcoma
			Dunning leukemia (rat)
			Hamster tumors
			Adenocarcinomas of the duodenum, pancreas, prostate, kidney, and small bowel
			Plasmacytoma No. 1
			Melanotic melanoma
			Cystadenocarcinoma of the liver No. 1

*Numbers in parentheses are optimal %ILS or tumor weight inhibition (TWI).

layed deaths after the time that the animals were killed. This problem can be circumvented largely by examination of tumor weights at doses that produce relatively little toxicity (up to the LD10) or minimal animal weight loss. Another way to circumvent this problem is to estimate tumor size by palpation rather than weight and to hold the animals for observation of latent toxic effects.

B. Drug Effect on Survival Time of Tumorous Host; Schedule and Route of Administration

The therapeutic efficacy of a drug, as initially emphasized by Marshall in antimalarial chemotherapy (24) and as subsequently demonstrated in a series of antitumor studies (25-28), may be markedly influenced by the schedule of administration of the drug. In addition to alteration of individual and total dosage, pertinent scheduling may encompass: (*a*) the total duration of treatment, (*b*) the total number of treatments, and (*c*) the interval between treatments. A broad range of information including these parameters of scheduling may be of great importance to the clinical investigator.

In addition to the studies currently conducted (table 1) (1), it is suggested that more extensive testing with tumors of interest could be incorporated in a comprehensive preclinical program. Generalized protocols could be established for such detailed schedule-dependency characterization. In these protocols the total interval (duration) of treatment could be varied, as well as the number of days of treatment for each of the total intervals of treatment. Also, for each day of treatment the number of individual treatments could be varied. For this type of protocol, dose-response studies are preferable in each instance for every experimental group. Because of the large number of required experimental groups it may be necessary to conduct the study in sequential experiments. Such adjustments may be made in accordance with the physical facility in the experimental laboratory, the number of personnel, the mice available, and other considerations. A systematic characterization could first involve determination of optimal treatment for a single day. Such optimal treatment could then be used as a positive standard for comparison with one of the multiple treatment schedules. The optimal therapy of the

best of these two schedules could then be employed as a positive standard against a third total interval of treatment, and so forth.

That the total duration of treatment may influence therapeutic effectiveness is illustrated in a study with methotrexate in the treatment of mice with advanced leukemia L1210 (fig 9) (29). It was found that when using daily injections of methotrexate for treatment of advanced disease it was advantageous to continue the treatment indefinitely. When the daily treatment was discontinued, the leukemic animals died, on the average, in 2-3 days. The result suggested that the failure to derive any benefit from discontinuation of the treatment resulted from an inability to obtain sufficiently extensive regression of advanced leukemia with regimens of treatment that are not lethal to the host.

Figure 10 illustrates an experiment in which a fixed total interval of treatment was used while the number of days of treatment was varied (12). The experiment involves a comparison of the therapeutic

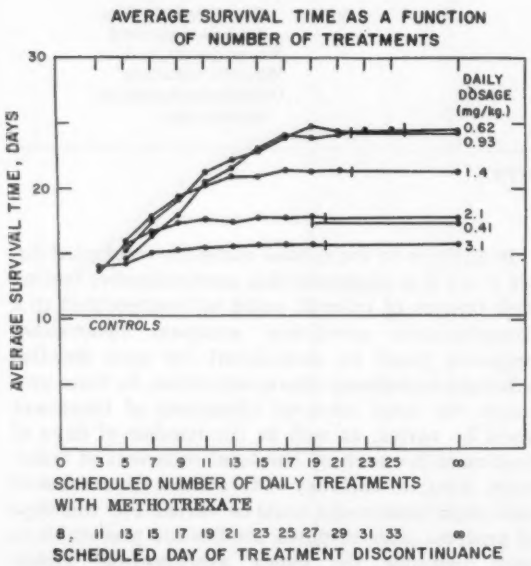


FIGURE 9.—Average survival time of leukemic mice as a function of the scheduled number of treatments with methotrexate. For each schedule of treatment the average survival time was estimated according to the actuarial procedure as described in the text. The abscissa corresponding to the vertical bar on each curve indicates the longest individual survival time observed during the course of treatment; the average survival time is not changed by scheduling treatments beyond the maximum individual survival time. Estimated average survival times are not shown for schedules extending beyond the maximum individual survival time observed during treatment. In some instances it was necessary to estimate the average survival time employing a conservative procedure [*from* ref 29].

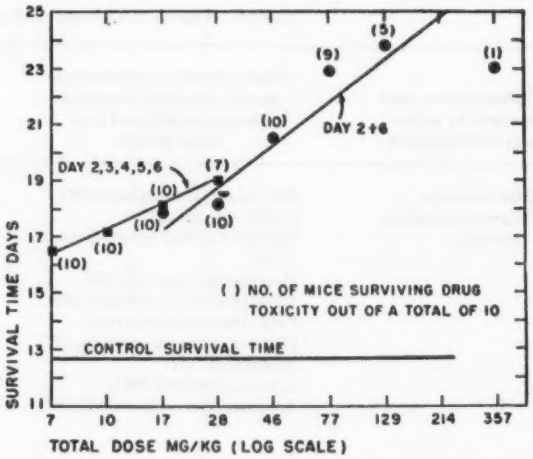


FIGURE 10.—Comparison of the antileukemic action of daily administration and spaced treatment with methotrexate (equal total interval, Days 2-6) [*from* ref 12].

effectiveness of daily administration of methotrexate on Days 2-6 following inoculation of leukemia L1210 as compared with treatment on Days 2 and 6. For the equal total interval of 5 days it was possible to employ a higher total dose of methotrexate with the spaced treatment (Days 2 and 6) as compared with daily treatment, and to thereby obtain a more extensive increase in survival time of the animals.

On another schedule involving continuous treatment initiated relatively early following leukemic inoculation, treatment with methotrexate every 4 days was considerably more effective than daily treatment or single treatment in extending the survival time of mice with early leukemia L1210 (fig 11) (27). The intermittent schedule with methotrexate has also proven to be more effective than daily treatment in remission maintenance of children with acute lymphocytic leukemia (30).

Skipper et al (31, 32) provided evidence that cytosine arabinoside exerts its activity during the S phase of the cell cycle and they demonstrated that ip treatment given every 3 hours per day on an intermittent schedule (Days 2, 6, 10, and 14 after leukemic inoculation) was an optimal schedule against the early stage of ip implanted leukemia L1210 in mice. These findings were confirmed in our laboratory, and an experiment in which the number of treatments per day of cytosine arabinoside was varied is illustrated in table 6 (33). In this experiment a fixed total interval of treatment with cytosine arabinoside was employed against early or advanced leukemia L1210 (Days 1-9 or Days 5-13). The number of treatments was varied (every 2, 4, or 8 days) and the number of treatments per day of drug administration was also

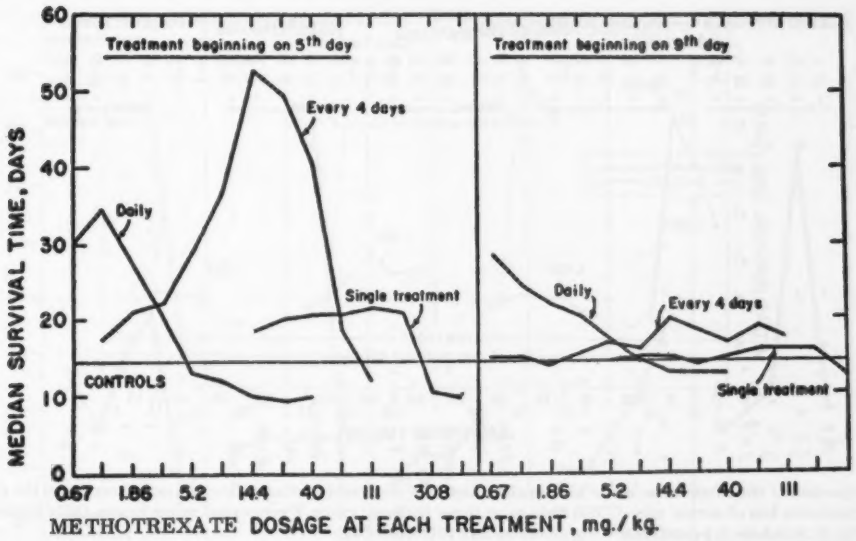


FIGURE 11.—Effect of schedule of treatment with methotrexate on the MST of mice with leukemia L1210. When treatment was initiated on the 5th day after tumor implant, the q4d schedule of treatment was most effective. When treatment was delayed until the 9th day, daily treatment became relatively more effective. The tumor inoculum was 1.13×10^5 cells per mouse, or 10 times that yielding 100% takes in the tumor titration [*from* ref 27].

varied (single treatment as compared with treatment every 3 hours per day). In this experiment injections of cytosine arabinoside administered ip to the leukemic mice every 3 hours for 24 hours (q3h/24h) on every second or fourth day starting 1 day after ip inoculation of 10^5 L1210 cells constituted the superior schedule; it was considerably more effective than single treatments given every second or fourth day. Against more advanced disease the schedule dependency was no longer evident.

The route of inoculation of the tumor and the route of administration of the drug may play an important role in the determination of drug effectiveness. It is therefore desirable that protocols be considered for sc, ip, and iv inoculation of the tumor, each with sc, iv, ip, or oral administration of the drug. An experiment in which daily treatment with 3′,5′-dichloromethotrexate, administered orally, was considerably less effective than the same schedule administered sc is illustrated in figure 12 (34). The

TABLE 6.—Cytosine arabinoside treatment of early and advanced leukemia L1210* [*from* ref 33]

	Days 1-9			Days 5-13		
Treatment schedule	Optimal dose (mg/kg/day)†	% ILS‡	60-Day S/T§	Optimal dose (mg/kg/day)†	% ILS‡	60-Day S/T§
Daily	39/1	89	0/8	23/1, 39/1	89	0/8
Every 2 days	833/1	178	1/8	833/1	128	0/8
q3h, every 2 days	40/8	222	3/8	67/8	133	0/8
Every 4 days	2320/1	100	0/8	2320/1	89	0/8
q3h, every 4 days	184/8	>422	4/8	184/8	122	0/8
Every 8 days	2320/1	67	0/8	2320/1	39	0/8
q3h, every 8 days	184/8	111	0/8	184/8	61	0/8

*10^5 ascites cells implanted ip; eight BDF_1 male mice (18-23 g) per group.

†Ip treatment was given over a wide range of logarithmically spaced doses. The maximally effective doses shown are expressed as total dose per day/No. of injections per day. For example, treatment with 5 mg/kg q3h/24h is expressed as 40/8.

‡Percent increase in median lifespan over controls. Median survival time of untreated controls = 9 days.

§No. of survivors/total treated.

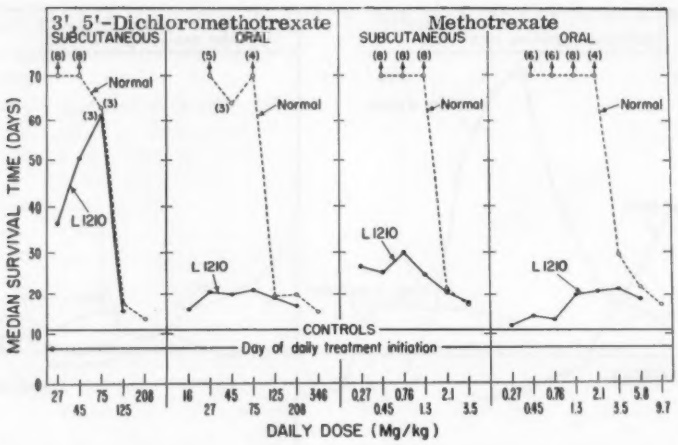


FIGURE 12.—Comparison of the effects of sc and oral administration of 3',5'-dichloromethotrexate and methotrexate on the survival of mice with systemic leukemia and of normal mice. CDBA male mice; 10 per leukemic group, 8 per normal mouse group. Daily treatments were given from Day 7 to 67. Numbers in parentheses = survivors on Day 70 [*from* ref 34].

influence of route of administration for cytosine arabinoside in the daily treatment of advanced leukemia L1210 is shown in figure 13 (35). The activity for sc, iv, and ip routes and the loss of activity on oral administration may be noted.

In another study it was observed that the advantage of the q3h/24h/q4d schedule for cytosine arabinoside observed when treatment is initiated early against ip implanted leukemia was not evident when the drug was administered orally (table 7) (33).

In the treatment of the Lewis lung carcinoma growing primarily in the lung following iv inoculation, adriamycin was therapeutically effective when administered iv and showed a loss of effectiveness when administered ip (36). Lewis lung carcinoma implanted sc was not sensitive to adriamycin regardless of the route of drug administration (36).

TABLE 7.—Oral cytosine arabinoside treatment of early leukemia L1210* [*from* ref 33]

Treatment schedule (Days 1-9)	Optimal dose (mg/kg/day)†	% ILS‡
Daily	108/1	61
Every 4 days	833/1	133
q3h, every 4 days	520/8	150

*10^5 ascites cells implanted ip; eight mice per test group and 16 mice in untreated control groups (female BDF_1 mice, 17-22 g).

†Oral treatment was given over a wide range of logarithmically spaced doses. The maximally effective doses shown are expressed as total dose per day/No. of injections per day.

‡Percent increase in median lifespan over controls. Median survival time of untreated controls = 9 days.

The stage of disease development may have an influence on the optimal scheduling. For this reason it would be important to conduct detailed schedule-dependency studies not only against early disease but also, with appropriate modification, against advanced stages of the disease. The alteration of optimal scheduling in accordance with stage of disease is illustrated in figure 11 (27). Although methotrexate on an every-fourth-day schedule was highly effective against early leukemia L1210, it was no more effective than a daily schedule against advanced systemic disease. In another example (table 6) (33), as cited above, the advantage observed on the q3h/24h/q4d schedule for cytosine arabinoside in the treatment of early leukemia L1210 was no longer evident when the disease was advanced.

There is considerable interest in the utilization of intermittent schedules of drug administration in the clinic. In order to provide more detailed information on the influence of intermittent schedules, experimental designs could be employed involving, for example, treatment daily, treatment every second day (1 day on, 1 day off), 3 days of treatment and 3 days off, and 9 days of treatment and 9 days off. For each schedule, treatment could be discontinued following varying durations. Where desirable this type of schedule can be employed for multiple treatments per day as compared with a single treatment for each day of treatment.

For these types of protocols, as for others discussed above, it would be important to conduct dose-response studies for each experimental group. Also, where there are a large number of required experimental groups it may be necessary to conduct the

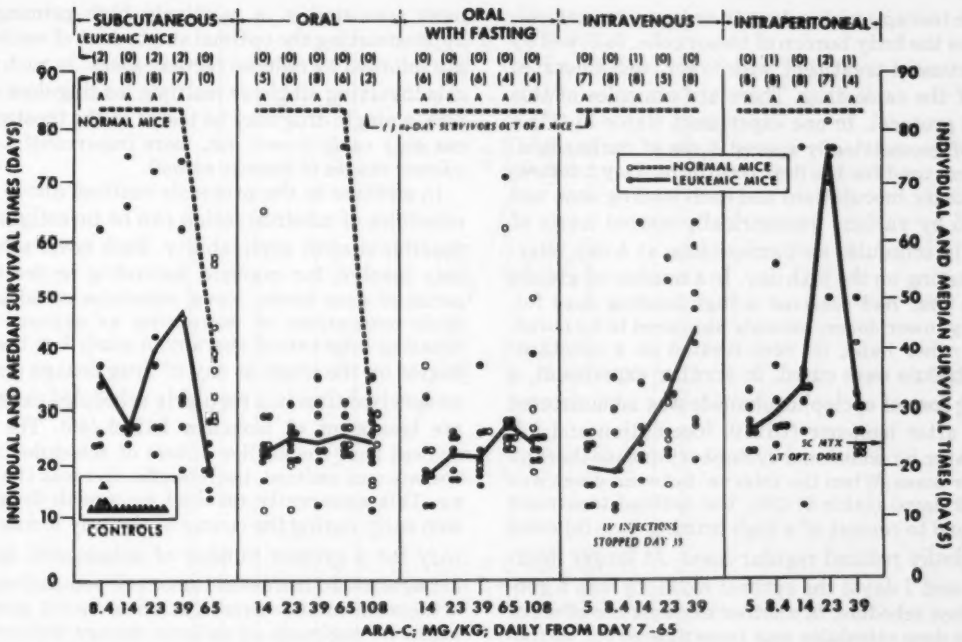


FIGURE 13.—Influence of route of administration of cytosine arabinoside (ara-C) on the MST and individual survival time of normal mice and mice inoculated sc with L1210 when injections were started 7 days after inoculation. Injections were terminated on Day 65 for all surviving mice. Eight $CD2F_1$ male mice (19-27 g) per treated group and 16 untreated controls were used [*from* ref 35].

study sequentially. An additional type of intermittent therapy protocol applicable to slow-growing tumors could involve drug administration on alternate weeks for varying total intervals. For each week of treatment the scheduled days of treatment and the number of treatments per day may also be varied.

With the various protocols different routes of tumor inoculation and drug treatment may be employed, and with appropriate modification treatment can also be administered against advanced rather than early disease.

One type of schedule with potential value for treatment of advanced disease involves heavy load-

TABLE 8.—Effect of loading-dose schedules of carbazilquinone on survival time in mouse leukemia L1210* [*from* ref 37]

Carbazilquinone (mg/kg, q4d, Day 5 to death)	MST† (days)						
	Initial dose of carbazilquinone on Day 1 (mg/kg)						
	2.86	2.00	1.40	0.98	0.69	0.48	0.33
2.86	8.5				10	10.5	12
2.00	11.5	17.5	18.5	17	13.5	17	19.5
1.40	9.5	60 (5)	25.5	26 (1)	18	20	20
0.98	24.5 (2)	23.5	16.5 (1)	18	18	16	14.5
0.69	60 (5)	21 (3)	18.5 (2)	15.5	14	13	13
0.48	21 (2)	22	17	13.5	13	13	12
0.33	14 (2)	23 (3)	16	13.5	13		10.5

*CDF_1 mice (19-25 g) were inoculated with 10^5 L1210 ascites cells ip, Day 0. There were 8 mice per group. MST of untreated control mice was 8.0 days. Cured mice survived longer than 90 days but were recorded as having lived 60 days. Treatment was ended on Day 40. The MST of groups of mice which received the constant dose schedules are joined by a diagonal line.

†Numbers in parentheses indicate the number of mice cured.

ing-dose therapy with a drug in order to dramatically reduce the body burden of tumor cells, followed by more extended treatment with lower, well-tolerated doses of the same drug. There are examples of this type of protocol. In one experiment (table 8) (37) a series of geometrically spaced doses of carbazilquinone were used for the first treatment (Day 1 following leukemic inoculation) and each loading dose was followed by various geometrically spaced levels of regularly scheduled carbazilquinone at 4-day intervals starting on the fifth day. In a number of groups of mice that had received a high loading dose followed by lower doses, animals appeared to be cured. On the other hand, no mice treated on a constant-dose schedule were cured. In another experiment, a priming dose of cyclophosphamide was administered 4 days after leukemic (L1210) inoculation and followed later by additional cyclophosphamide therapy at lower doses. When the interval between doses was short (2 days) (table 9) (38), the optimal treatment was found to consist of a high priming dose followed by markedly reduced regular doses. At longer intervals (5 and 7 days) the optimal modality was a constant-dose schedule. In another example the effect of priming-dose schedules was investigated for methotrexate treatment of mouse leukemia L1210 (39). In this study a comparison was made of the influence of loading-dose schedules and constant-dose schedules of methotrexate on survival time (tables 10 and 11) (39). It was indicated that a priming dose of methotrexate may lower the tumor cell population sufficiently for subsequent low doses of methotrexate to kill tumor cells more effectively. In this investigation the interval between doses was also altered (table 11). On each of the schedules the optimum treatment consisted of a relatively high priming dose approximating the optimal single dose of methotrexate followed by reduced regular doses. In such protocols involving single or multiple loading-dose schedules, a single drug may be tested in the treatment of not only early tumor but, more importantly, of advanced stages of disease as well.

In addition to the protocols outlined above, novel schedules of administration can be investigated for possible clinical applicability. Such novel schedules may involve, for example, ascending or descending series of dose levels. Novel schedules could also include comparison of decreasing as opposed to increasing intervals of therapy. A study has been conducted on the effect of day of drug dosage omission on survival time in a regularly scheduled methotrexate treatment of leukemia L1210 (40). The study showed that the earlier a dose of scheduled methotrexate was omitted, the less effective was the therapy. This apparently resulted since with drug omission early during the course of therapy it was necessary for a greater number of subsequent doses to act against an increased tumor cell population.

Included in the investigation of novel schedules might be regimens of tailored dosage and schedule based on body weight changes, white blood cell count and extent of bone marrow suppression and recovery, and alteration of biochemical parameters or markers reflecting disease status.

C. Relation of Dose-Response Curves in Treated Tumor-Bearing Mice to Dose-Response Curves in Normal Mice

It is instructive to compare the dose-response curves in tumor-bearing animals treated with the

TABLE 9.—Priming-dose cyclophosphamide schedules: comparison of the influence of loading-dose and constant-dose schedules of cyclophosphamide on survival time* [*from* ref 38]

q2d dose of cyclophosphamide from Day 6 to death (mg/kg)	MST (days)									
	Initial dose of cyclophosphamide, Day 4 (mg/kg)									
	337.5	225	150	100	66.7	44.4	29.6	19.7	13.1	8.7
150			9.5	10	10	11.5	11	12	11	11
100		12.5	14.5	19.5	18.5	19	21	14	11.5	14.5
66.7	10	24	23.5	21	18.5	14	13	12	11.5	10.5
44.4	22.5	22.5	22.5	21	16.5	12	10.5	10.5	10	10.5
29.6	23	19	17.5	15	14	12	10.5	10	10	10
19.7	26.5	18.5	16.5	14.5	12.5	11.5	10	10	9.5	9.5
13.1	10†	19.5	16	13	12	11	10	9	9	9
8.7	28.5	18	15	13	12	11	9.5	9	9	9

*CDF_1 male mice weighing 19-25 g were injected ip with 10^5 L1210 ascites tumor cells on Day 0. MST of tumor control mice was 8 days. There were 8 mice per group. The MSTs of groups of mice on constant-dose schedules are joined by a diagonal line.

†5 of 8 mice in this group died of toxicity by Day 10.

TABLE 10.—Comparison of the influence of loading-dose schedules and constant-dose schedules of methotrexate on survival time* [*from* ref 39]

Daily dose of methotrexate from Day 3 to death (mg/kg)	MST (days, range)											
	Initial dose of methotrexate, Day 2 (mg/kg)											
	100	60	36	21.6	12.96	7.77	4.66	2.80	1.68	1.01	0.60	0.36
2.80											10 (9-12)	11 (8-14)
1.68	7 (6-8)	13 (6-19)	9 (6-14)	10 (6-14)	13 (10-30)	12.5 (9-20)	13 (9-17)	9 (8-19)	12 (10-16)	17.5 (10-27)	14.5 (11-18)	14 (10-17)
1.01	7.5 (6-20)	10.5 (7-29)	14.5 (7-25)	11 (6-29)	17.5 (11-27)	16.5 (7-29)	19.5 (13-23)	16.5 (13-23)	19 (14-21)	19‡ (15-23)	13.5 (11-20)	19 (14-23)
0.60	14 (6-23)	8.5 (6-23)	22 (14-29)	25† (11-27)	19.5 (17-26)	20.5 (11-30)	21.5 (12-25)	20 (16-23)	18 (9-35)	18 (14-34)	17.5 (15-20)	16 (15-18)
0.36	7 (6-19)	19 (9-23)	18.5 (9-20)	17 (9-21)	18 (16-23)	17 (16-27)	18 (15-20)	15 (12-19)	15 (12-26)	16 (13-19)	14 (11-16)	13 (13-14)
0.22	15.5 (6-19)	18 (6-20)	16 (10-18)	15 (14-16)	15 (14-18)	14.5 (13-20)	14 (13-15)	13 (11-14)				

*CDF_1 male mice weighing 18-25 g received ip injections of 10^5 L1210 ascites tumor cells on Day 0. The MST of untreated control mice was 8 days, range, 8-9 days. There were 8 mice per group. The MSTs of groups of mice on constant-dose schedules are joined by a diagonal line.

†Maximum MST, all dose schedules used.

‡Maximum MST, constant-dose schedules only.

TABLE 11.—Comparison of the influence of loading-dose schedules and constant-dose schedules of methotrexate on survival time* [*from* ref 39]

Interval between doses of methotrexate (days)	Optimal constant-dose results		Optimal loading-dose results		
	Optimal constant dose of methotrexate beginning on Day 2 (mg/kg)	MST (days)	Initial loading dose of methotrexate on Day 2 (mg/kg)	Subsequent constant dose of methotrexate (mg/kg)	MST (days)
1	1.01	19 (15-23)†	21.6	0.6	25 (11-27)
2	4.66	22.5 (18-28)	60	2.8	25 (7-30)
3	12.96	18 (12-21)	60	7.77	21.5 (7-26)
4	21.6	16 (10-21)	36	12.96	20 (15-25)
5	21.6	16 (12-17)	60	21.6	19.5 (6-21)

*CDF_1 male mice weighing 18-25 g received ip injections of 10^5 L1210 ascites tumor cells on Day 0. MST of untreated control mice in each experiment was 8 days.

†Nos. in parentheses = range of survival time.

drug to the corresponding dose-response curves in normal animals. For an active compound the dose-survival time curve in the tumorous animals in general shows a progressive increase in survival time with an increasing dose. A maximum increase in survival time is achieved at an optimal dosage following which the survival time diminishes as the toxicity for the host becomes more evident. With some drugs the dose-response curve in normal animals may correspond essentially with the descending arm of the dose-response curve for the tumorous animals. In such cases, the maximum increases in survival time obtained at the apparent "optimal dosage" for the tumorous animal would coincide with the time of death in the normal animals, indicating that the toxicity of the drug for the host is severely limiting. It would be recommended, in such cases, that dosages well below the apparent "optimal dosage" be employed for therapy. Such limitation of toxicity for the host was observed in experiments with 5-fluorouracil (fig 14) (41). Even where drug toxicity for the host is so severely limiting it may still be possible to improve the therapeutic response by relative diminution in the toxicity of the drug by means of alteration of the schedule of administration or by administration of a second drug. With other drugs or therapeutic regimens the toxicity curve relating survival time to dosage may be considerably higher than the curve for the treatment of the tumorous animals. This situation occurred during sc and oral treatment of leukemia L1210 with methotrexate and during oral administration of 3′,5′-dichloromethotrexate (fig 12) (34). In such instances the limitation of therapeutic response may be attributable to an increase in toxicity for the tumorous animals and/or a reduction in therapeutic effect. In any event, the emphasis in such situations would be to explore means for nutritional or other types of fortification of the tumorous host, means for improved drug distribution and transport, and so forth. In the study with 3′,5′-dichloromethotrexate it was suggested that with oral administration the drug failed to reach the tumor site in a concentration sufficient to achieve its maximum potential. This is in line with the observation of Rall et al (42) that the maximum plasma concentration of 3′,5′-dichloromethotrexate after oral administration to dogs and humans was substantially lower than the plasma concentration after parenteral injections of the drug. Also it was observed (43) that the capacity of both 3′,5′-dichloromethotrexate and methotrexate

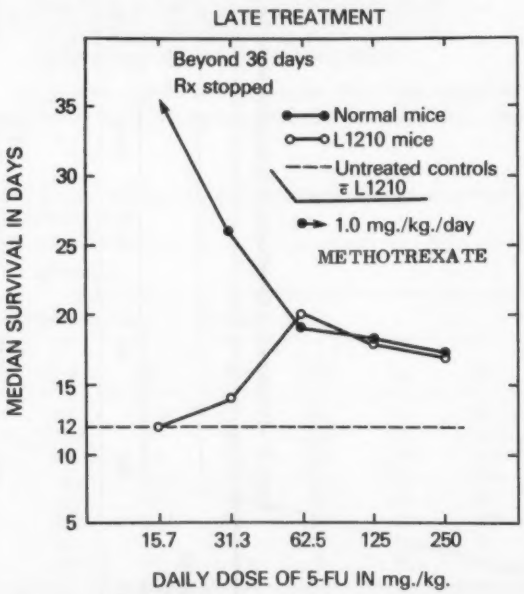


FIGURE 14.—Comparison of MSTs of normal and leukemic mice with treatment beginning on Day 10, including comparison with an optimal dose of methotrexate (1.0 mg/kg/day) [*from* ref 41].

to inhibit the incorporation of ^{14}C-formate into the acid-soluble adenine of leukemic spleens of mice is markedly reduced with oral administration.

D. Drug Effect on Tumor Cell Kill and on Cure of Animals

The extent of destruction of tumor cells may also be determined. For solid tumors, such as sc or im tumors, a dose-response study can be conducted including doses well above the LD50 (2 × LD50 or higher) and ranging to relatively nontoxic doses. The dose level at which total eradication of the local tumor, or a designated percentage reduction relative to untreated controls, is achieved can be determined by bioassay (assuming that the drug is sufficiently potent for this purpose). Alternatively, the tumor can be bioassayed at various times following treatment over a tolerated dose range to determine the proportion of cells remaining. The tumor may also be examined by dye-exclusion techniques or by histologic methods for surviving cells.

For metastasizing tumors, including leukemia, bioassay can be conducted of local tumor, blood, and various tissues. The bioassay procedure may involve growth on transfer to syngeneic recipients and also the spleen colony assay.

For a tumor growing at the intracranial site, brain tissue may be bioassayed following parenteral administration of the drug.

After drug administration to an ascites tumor, a direct count can be made of the surviving tumor cells, or the number of residual tumor cells can be determined by bioassay. Biochemical measurements can be related to other measurements of the extent of reduction of the tumor cell population. For the various biologic models the time course of inhibition and recovery of DNA and RNA levels can be determined based on ^{3}H-thymidine and ^{14}C-uridine incorporation. ^{125}I-labeled IUdR incorporation into cells can also be determined as a measure of DNA synthesis and cell replication. Additional studies may be conducted on the ability of the drug to reach the target tumor cells, to enter the cells, and so forth. The extent of incorporation of radiolabeled candidate drug into the cells can also be determined.

In the above models involving single or short-term therapy, markedly cytotoxic agents that are not cell

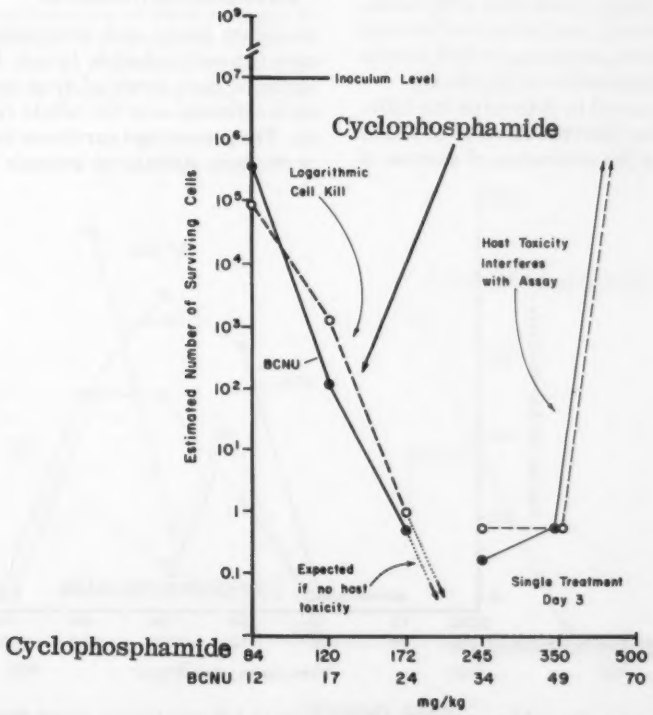


FIGURE 15.—Estimation of the number of surviving cells after 1 treatment with BCNU or cyclophosphamide. Treatment given 3 days after the sc inoculation of 10^7 L1210 splenic cells. The relationship between dosage and surviving leukemic cells is shown until host toxicity interferes with the assay. The calculations in this experiment are based on a generation time of 0.9 days [*from* ref 45].

cycle specific in their action may destroy a high percentage of tumor cells. Agents that inhibit cell division but are not markedly cytotoxic, exerting their primary action at a specific locus in the cell cycle such as S phase, will demonstrate low cell-kill capacity but may be highly active on multiple-treatment schedules. Where the influence of the schedule of therapy is under investigation, a comparison can be made of cell-kill capacity of a drug at maximum tolerated dosage for each schedule utilized.

In addition to the procedures outlined above for measurement of extent of destruction of tumor cells, in which histologic, bioassay, and biochemical procedures are employed for both local and systemic disease, the survival time and percentage cure of the animals can be determined. Once the generation time of the tumor has been determined it is possible to estimate the percentage tumor cell kill from the increase in survival time over controls (44). This is illustrated in figure 15 (45) where the number of surviving leukemic cells is estimated based on a hypothetical generation time of 0.9 days, following treatment with BCNU or cyclophosphamide.

It is also possible to measure the percentage of survivors at a series of inoculum levels for varying dosages of drug. In one study conducted with methotrexate, a linear relationship was observed between the leukemic inoculum level resulting in 50% deaths and the dosage of the drug employed (fig 16) (46).

Protocols may be employed to determine the influence of scheduling on the total eradication of tumor cells. This would involve the utilization of a series of

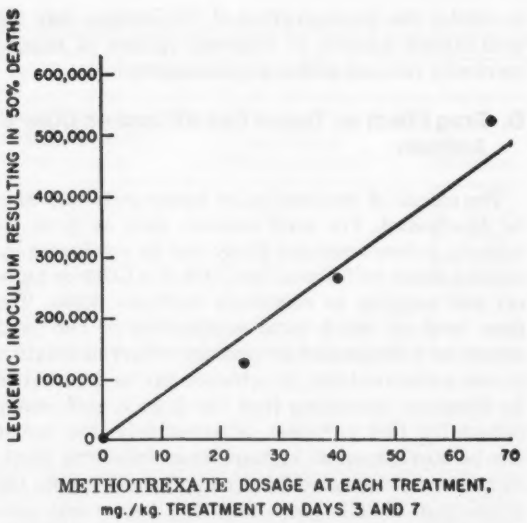


FIGURE 16.—Relationship between level of treatment with methotrexate and the size of the leukemic inoculum (cells per mouse) required to yield 50% mortality (EI50). Early deaths from drug toxicity are excluded in computing the percentage mortality resulting from the leukemic inoculum. Without treatment, the EI50 was about 4000 cells [*from* ref 46].

inoculum levels and determination of percentage cure for each schedule tested. In a detailed study a series of dose levels of drug would be employed for each schedule over the whole range of inoculum levels. The percentage survivors may be measured at 60 or 90 days. Additional animals could be included for

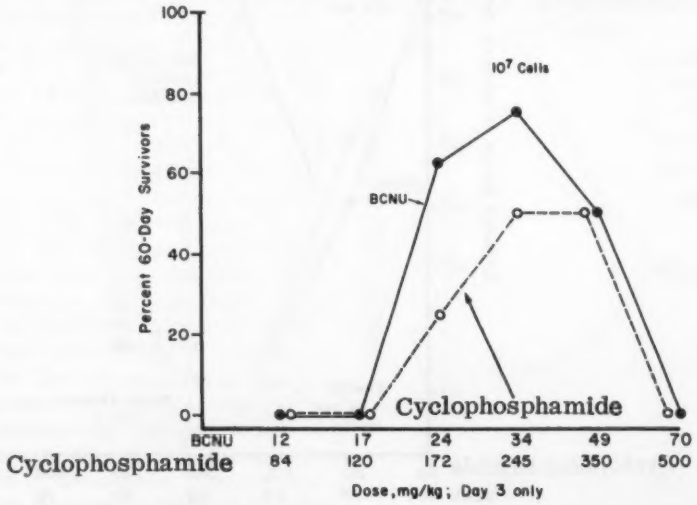


FIGURE 17.—Dose-response plot; comparison of the percentage of 60-day survivors following 1 treatment with BCNU or cyclophosphamide in mice with leukemia L1210. Eight BDF_1 mice per treated group and 16 untreated mice were inoculated sc with 10^7 L1210 splenic cells on Day 0. One sc injection was given on Day 3. All 60-day survivors were without palpable tumor. The untreated controls died on Days 9-11 with an MST of 10 days [*from* ref 45].

bioassay at stated intervals to determine the extent of destruction of tumor cells, the duration of remission, and the course of relapse. Also, representative survivors may be bioassayed to determine whether there is any residual disease, and other survivors may be rechallenged to determine the extent to which animals have become refractory to tumor growth.

In measuring the percentage of survivors it may be noted that the toxicity of the drug for the host may become limiting, as was noted in studies in which the increases in survival time of the animals are determined. This is illustrated for cyclophosphamide and for BCNU (fig 17) (45). That the inoculum level may influence therapeutic effectiveness is illustrated in an experiment with BCNU (fig 18) (45) in which the percentage of survivors among animals that received 10^5 or 10^6 cells of leukemia L1210 was higher than that observed for animals that received 10^7 cells. By employing the "curability" system it is possible to compare the relative effectiveness of drugs with respect to their ability to produce survivors and this is illustrated in figure 19 (45). In this experiment, using single treatments, BCNU and cyclophosphamide were shown to be more effective than cytosine arabinoside, which in turn was more effective than 6-mercaptopurine or methotrexate in producing cures in animals with leukemia L1210.

It should be pointed out, as illustrated in figure 20 (45), that the schedule of therapy may play an im-

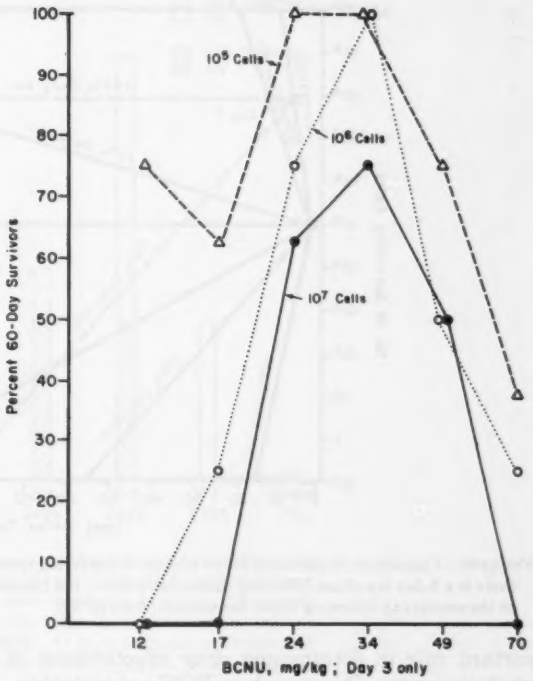


FIGURE 18.—Influence of the size of the leukemic inoculum on the ability of BCNU to produce survivors [*from* ref 45].

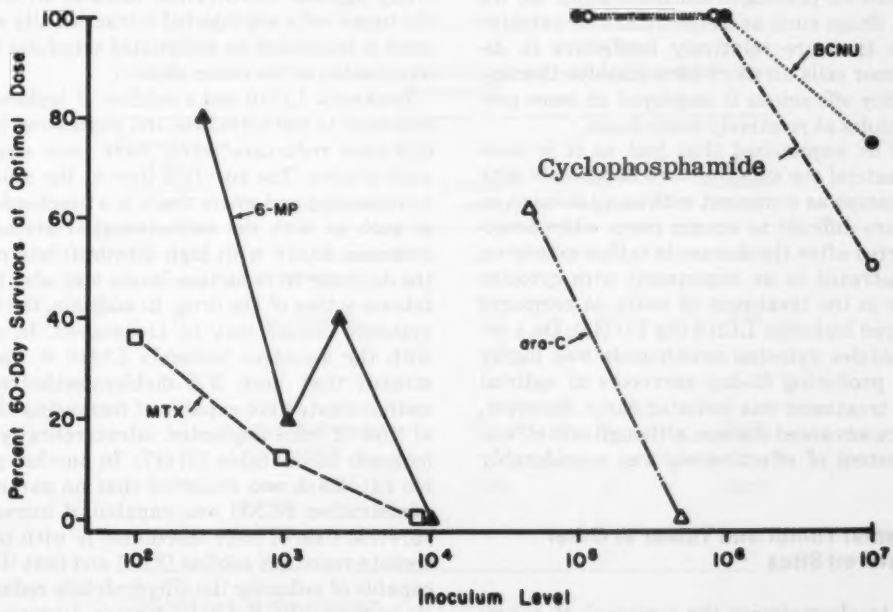


FIGURE 19.—Comparison of drugs with respect to their ability to produce survivors. Data are from separate experiments. BCNU and cyclophosphamide were given sc on Day 3 only after sc inoculation of L1210 splenic cells; cytosine arabinoside (ara-C) and 6-MP were given sc on Day 3 only after sc inoculation of L1210 ascites cells. Methotrexate (MTX) was given on Days 2 and 6 [*from* ref 45].

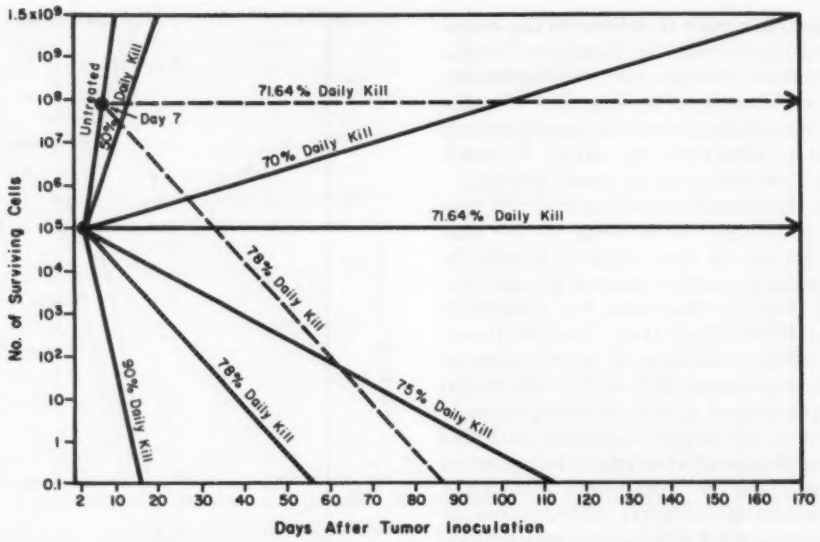


FIGURE 20.—Calculated relationship of the number of surviving tumor cells with varying percentage kill on daily therapy. It is assumed that there is a 2-day lag phase following tumor inoculation, and the generation time of the tumor cells is 0.55 days. Daily treatment is initiated on the second day following tumor inoculation [*from* ref 29].

portant role in determining drug effectiveness in producing cures. Drugs such as BCNU or cyclophosphamide that are highly effective in destroying tumor cells on short-term massive therapy may become too toxic on prolonged administration. On the other hand, drugs such as methotrexate or cytosine arabinoside that are relatively ineffective in destroying tumor cells on short-term massive therapy may be highly efficacious if employed on more prolonged schedules at relatively lower doses.

It should be emphasized that just as it is more difficult to extend the survival time of animals with advanced disease as compared with early disease, so too it is more difficult to obtain cures when treatment is started after the disease is rather extensive. This is illustrated in an experiment with cytosine arabinoside in the treatment of early as compared with advanced leukemia L1210 (fig 21) (45). On a series of schedules cytosine arabinoside was highly effective in producing 65-day survivors at optimal doses when treatment was initiated early. However, against more advanced disease, although still efficacious, the extent of effectiveness was considerably reduced.

E. Intracranial Tumor and Tumor at Other Sequestered Sites

In order to characterize the potential of a new drug for treatment of intracranial tumor or tumor at other sequestered sites it is important to determine whether the drug can reach the tumor cells. For the treatment of intracranial tumor, determination can be made as to whether a drug crosses the blood-brain barrier and whether it exerts therapeutic activity against intracranial disease. In one system the tumor cells are injected intracranially and treatment is instituted on designated schedules following inoculation of the tumor cells.

Leukemia L1210 and a subline of leukemia L1210 resistant to methotrexate and possessing high dihydrofolate reductase levels have been employed in such studies. The survival time of the animals may be measured and where there is a biochemical marker such as with the methotrexate-resistant line of leukemia L1210 with high dihydrofolate reductase, the decrease in reductase levels may also reflect cytotoxic action of the drug. In addition, the brain and systemic tissues may be bioassayed. In one study with the sensitive leukemia L1210 it was demonstrated that both 3′,5′-dichloromethotrexate and methotrexate were capable of increasing the survival time of mice implanted intracerebrally (ic) with leukemic blood (table 12) (47). In another study (table 13) (48) it was observed that on parenteral administration BCNU was capable of increasing the survival time of mice inoculated ic with the methotrexate-resistant subline (FR9) and that it also was capable of reducing the dihydrofolate reductase levels in the brain. For solid tumors, tumor cells could be inoculated directly into the brain and the influence of drug treatment on the extent of growth of

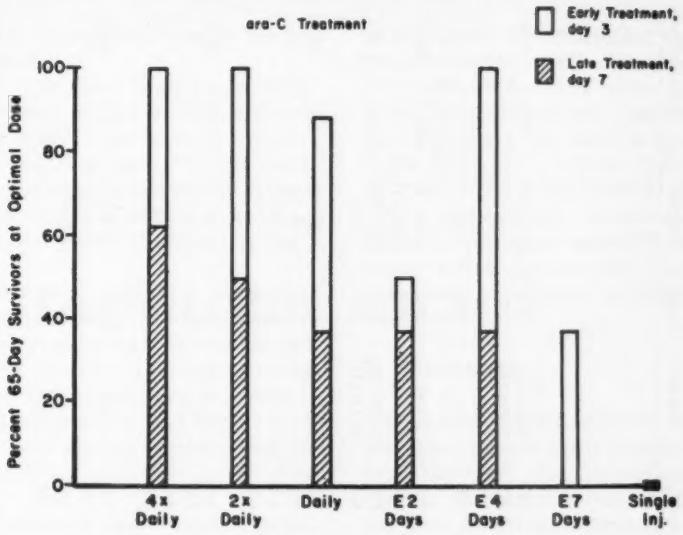


FIGURE 21.—Influence of day of treatment initiation and schedule of treatment on the ability of cytosine arabinoside (ara-C) to produce survivors. BDF_1 male mice, 8 per treated group and 16 untreated controls, were inoculated sc with L1210 splenic cells. Sc injections were terminated on Day 63. In the early treatment experiment the untreated controls died on Days 10-15; MST = 11 days. In the delayed treatment experiment the untreated controls died on Days 11 and 12; MST = 11 days [*from* ref 45].

TABLE 12.—Effect of drug therapy on mice implanted ic with L1210 leukemic blood* [*from* ref 47]

Concentration of blood inoculum† (%)	Drug‡	Treatment (mg/kg)	MST (days)	Range of deaths (days)
10	3′, 5′-dichloromethotrexate	50	41	15-68
10	Methotrexate	0.75	19	15-30
10	None		12	11-12
1	3′, 5′-dichloromethotrexate	50	52	14-66
1	Methotrexate	0.75	28	23-29
1	None		12	12-13

*DBA/2J mice, 10 mice per group.

†All inocula diluted with sterile saline and injected ic, 0.05 ml/mouse.

‡Drug therapy was started on the 7th day after mice were implanted ic and was administered sc daily in axillary region.

TABLE 13.—Effect of treatment with cyclophosphamide on the growth of intracranially inoculated methotrexate-resistant leukemia L1210 (FR-9) as determined by pathologic examination, retransplantation of tissues, and enzymatic assay* [*from* ref 48]

				Pathology					Transplantation						
					Infiltration in—					Spleen		Brain		Dihydrofolate reductase activity§	
Group	Treatment (days)	MST in days (range)	Day of sacrifice	Degree of infiltration†	Spleen	Liver	Dura	Brain (arachnoid)	Concentration‡	No. of takes/total	Median day of death (range)	No. of takes/total	Median day of death (range)	Spleen	Brain
1	None	8(8-9)	—												
2	4	15(9-17)	—												
3	None	—	4	0 to ±	6/6	3/6	1/6	1/6	1:10	3/3	14(12-15)	3/3	15(13-15)	39.8	30.8
				+ to ++		3/6	1/6	1/6	1:100	3/3	14(14-15)	1/3	17		
				+++ to ++++			4/6	4/6	1:1000	3/3	16(16-18)	0/3	—		
									1:10,000	0/3	—	0/3	—		
4	None	—	7	0 to ±					1:10	3/3	8(8-8)	3/3	11(10-11)	2980.0	243.0
				+ to ++					1:100	3/3	10(9-11)	2/2¶	11(11-11)		
				+++ to ++++	3/3	3/3	3/3	3/3	1:1000	3/3	12(10-13)	3/3	14(13-14)		
									1:10,000	3/3	14(13-14)	1/3	16		
5	4-6	—	7	0 to ±	3/3	3/3	3/3		1:10	3/3	12(12-17)	3/3	11(11-12)	56.1	129.0
				+ to ++					1:100	0/3	—	3/3	14(14-14)		
				+++ to ++++				3/3	1:1000	0/3	—	2/3	10(14-24)		
									1:10,000	0/3	—	0/2¶	—		
6	4-10	—	11	0 to ±	3/3	3/3	2/3	1/3**	1:10	2/3	22.5(15-30)	3/3	11(11-11)	20.5	280.0
				+ to ++			1/3		1:100	0/3	—	3/3	14(12-14)		
				+++ to ++++				2/3	1:1000	0/3	—	3/3	17(16-17)		
									1:10,000	0/3	—	1/3	15		
7	4-13	—	14	0 to ±	3/3	3/3	3/3		1:10	1/3	15	3/3	12(12-13)	18.5	298.0
				+ to ++					1:100	0/3	—	3/3	12(12-14)		
				+++ to ++++				3/3	1:1000	0/3	—	3/3	16(16-16)		
									1:10,000	0/3	—	0/3	—		

*Tumor inoculum: heart blood from a mouse with resistant subline of leukemia L1210 (FR-9) diluted 1:10 with normal saline, inoculated ic, 0.05 ml/mouse. Treatment: cyclophosphamide diluted in normal saline, injected sc daily, 75 mg/kg/day from Day 4 on schedules indicated.

†0 to ± = little to no leukemic infiltration; + to ++ = moderate leukemic infiltration; +++ to ++++ = extensive leukemic infiltration.

‡Pooled brain or spleen of 3 mice in normal saline.

§m μmols of dihydrofolate reductase per mg of protein per hour at a temperature of 32°C.

¶Accidental deaths not included.

**The inoculation may have been unsuccessful for this mouse.

intracranial tumor and the survival time of the animals could be determined.

Neurogenic tumors that have been employed include ependymoblastoma and glioma injected directly into the brain (49) and a Rous sarcoma virus-induced gliosarcoma in beagle puppies (50). With these tumors, too, treatment may be parenteral on optimal schedules to determine extent of inhibition of tumor growth in the brain and survival time of the animals.

It is a general procedure to radiolabel the drug of interest and to determine whether the label appears in the brain. Such observations can be coupled with studies employing the therapy models to provide evidence as to whether the labeled drug is present in the brain in a pharmacologically active form. Inhibition of intracranial tumor growth suggests that the initial drug or an active metabolite does indeed reach the tumor site.

When the drug is effective against intracranial tumor it is possible to conduct additional experiments to determine the relative effectiveness of the therapy against intracranial tumor as compared with tumor in other sites. Such a comparative study using ic and sc tumor was conducted by Kline et al (table 14) (51) for methotrexate, cytosine arabinoside, BCNU, and 3′-bromo-5′-chloromethotrexate. In each instance the drug was less effective against ic disease than against sc disease in increasing the survival time of the animals. For cytosine arabinoside the reduced effectiveness against ic leukemia was also reflected in more rapid deaths on bioassay. For tumors such as the Lewis lung carcinoma the effectiveness of a drug in the therapy of intracranial tumor could be compared with that observed for treatment of local sc or im tumor and metastatic tumor in the lung.

Antitumor agents may be evaluated with respect to their ability to prevent the occurrence of intracranial disease. The systemic disease may be treated with the drug of interest on an optimal schedule at a series of doses and the brain may be bioassayed during the course of therapy in order to determine whether intracranial infiltration of tumor has occurred. Also, pathologic studies of the brain can be performed. In one investigation (52) it was observed that with continuing treatment of parenteral leukemia L1210 with methotrexate, progressive intracranial infiltration and growth of leukemic cells occurred. It was considered that this observation furnished experimental evidence that could account for the apparent increase during the last decade of the meningeal leukemia syndrome that has occurred in humans with acute leukemia and who have been treated with chemotherapeutic agents. The increase of meningeal leukemia has been attributed to both an increase in the lifespan of the patient with acute leukemia as the result of drug therapy and the relative ineffectiveness of many systemically administered chemotherapeutic agents against leukemic cells that reach the brain or cerebrospinal fluid side of the blood-brain barrier. Both of these conditions obtained in the reported study with methotrexate.

New models should be developed to evaluate the ability of antitumor agents to reach and exert therapeutic action against bone metastasis since this represents a serious problem in clinical cancer treatment.

F. Resistance

The primary limiting factor in the employment of antitumor agents is the toxicity of the drug for the host. However, with increasing usage of drugs of various structural categories and more prolonged and more effective therapy, the origin of tumor cell resistance also constitutes a notable obstacle to successful chemotherapy.

The rapidity with which resistance to a drug occurs can be determined readily with standard experimental tumor systems. With L-asparaginase it was observed that resistance to therapy against leukemia L5178Y occurred during one generation of treatment (53). Resistance to cytosine arabinoside in the treatment of leukemia L1210 has also been observed to occur relatively rapidly (54). In other instances it has been necessary to continue to treat over several transplant generations in order for drug resistance to occur (55). The origin of resistance may be influenced by the drug dosage, the schedule and route of administration, and the tumor burden as determined by the inoculum level of tumor cells employed and the extent of progression of the disease at the time that treatment was started. A new drug can be characterized with respect to the influence of such factors and comparisons can be made with established clinically active drugs.

Once tumor cell resistance has occurred, investigations can be conducted as to its persistence by following the time course of reversibility of the tumor cell population to sensitivity in untreated animals.

After the occurrence of resistance to the drug being utilized in therapy, it is necessary to make the important decision as to which drug should be chosen for subsequent treatment. From this point of view, it is important to define the cross-resistance characteristics of sublines of tumors that have become resistant to the drug being employed. A reference chart on cross resistance among anticancer agents published by Skipper et al (56) is presented in figure 22. Such reference charts should be maintained, with the continuing addition of new data on

TABLE 14.—Effect of drug therapy on mice inoculated ic or sc with leukemic L1210 blood [*from* ref 51]

Expt No.	Drug	Sc therapy starting Day 3*	Ic tumor inoculation†			Sc tumor inoculation†		
			Optimal dose (mg/kg)	MST (days)	Individual survival time‡ (days)	Optimal dose (mg/kg)	MST (days)	Individual survival time‡ (days)
1	Cytosine arabinoside	Daily	23.0	40	28,29,36,38,42,69,70, >90	23.0	>90	53, 62, 85, >90(5)
	Methotrexate	Daily	0.65	17.5	15(2), 17(2), 18, 19, 20(2)	0.65	28	22,27(2),28(2),37, 41,50
	Controls			8	8(11), 9(4), 10		10	9(5), 10(8), 12(3)
2	Cytosine arabinoside	Daily	14.0	34.5	27,31(2), 33, 36(2), 39, 48	14.0	>76	>76(8)
	Cytosine arabinoside	Day 3 only	833	18	15,17(2), 18(3), 20, 21	833	18	16(2), 17, 18(3), 19(2)
	BCNU	Daily	3.0	34.5	18, 26, 30, 34, 35, 37, 39, 44	3.0	52.5	34, 42(2), 45, 60, >76(3)
	BCNU	Day 3 only	23.0§	33	11, 18, 29, 33(2), 35(2), 36	23.0	>76	>76(8)
	3'Bromo-5'-chloromethotrexate	Daily	23.0	36	20(2), 31(2), 41, 42(2), 45	65.0	>76	21, 29, 65, >76(5)
	Methotrexate	Daily	1.08	22	14, 18, 21, 22(2), 25, 26, 27	0.23	>76	>76(8)
	Controls			10	10(9), 11(4), 12, 13(2)		12	11(4), 12(6), 13(2), 17(2), 20(2)

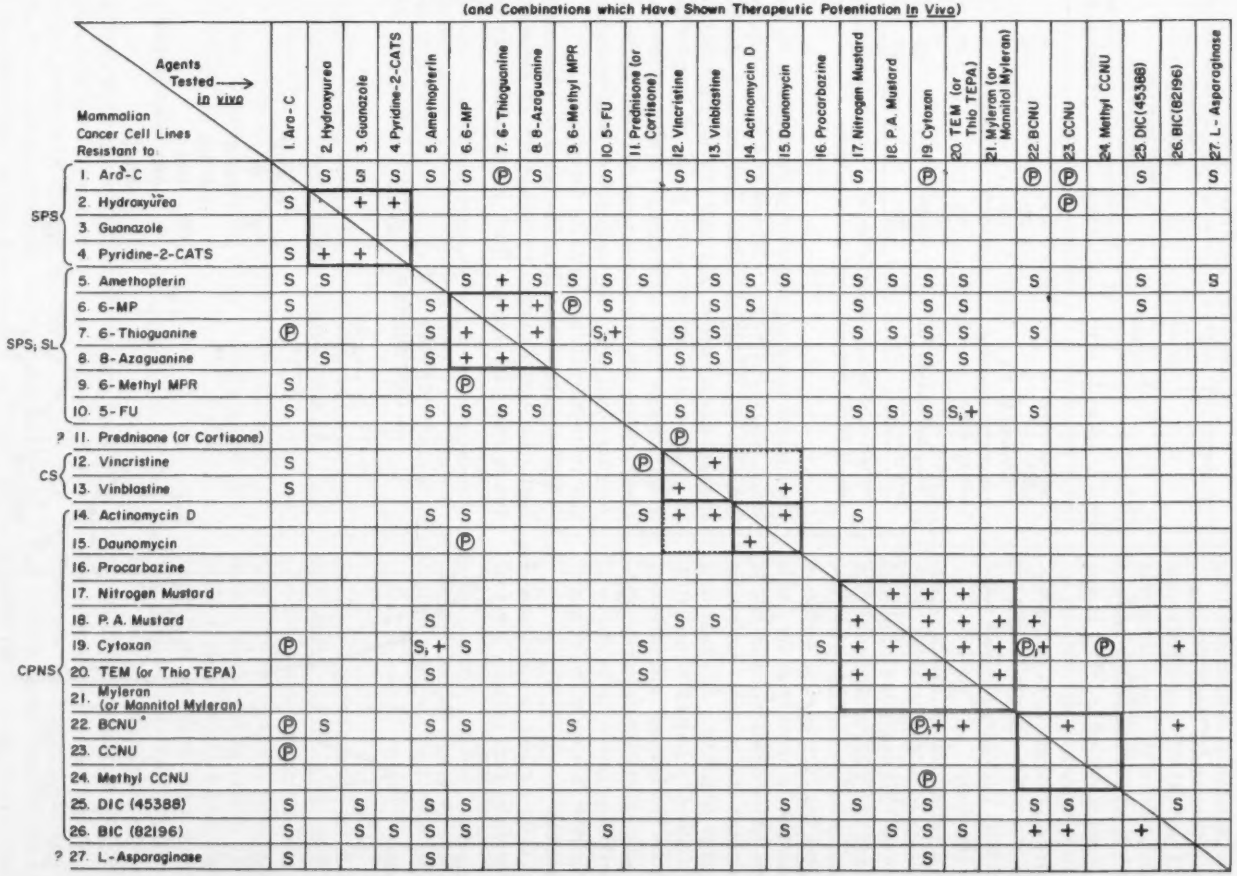
*Treatment was administered over a wide range of logarithmically spaced doses starting 3 days after tumor inoculation. The most effective dosage level for each treatment regimen is shown. Injections were stopped on Day 57 in expt 1 and on Day 52 in expt 2. Expt 1 was terminated on Day 97; expt 2 was terminated on Day 76.

†CDBA (expt 1) and BDF_1 (expt 2) male mice, 18-25 g (8 mice/test group), were inoculated ic with diluted leukemic blood with 0.05 ml/mouse, or sc in the right hind leg with 0.2 ml/mouse. Cellular concentrations of experimental inocula: expt 1 (ic, 262,500/0.05 ml; sc, 1,050,000/0.2 ml); expt 2 (ic, 170,000/0.05 ml; sc, 680,000/0.2 ml).

‡Figures in parentheses indicate number of mice. Survivors inoculated sc showed no evidence of palpable tumor.

§With a single injection of BCNU (39.0 mg/kg) 5 of 8 mice died on Days 14-20; 3 mice were surviving on Day 76.

QUICK REFERENCE CHART ON CROSS RESISTANCE BETWEEN ANTICANCER AGENTS
(and Combinations which Have Shown Therapeutic Potentiation In Vivo)



+ = Cross resistance. S = Not cross resistant; resistant line was as sensitive as or more sensitive than the parent tumor.

Ⓟ = Therapeutic potentiation demonstrated in vivo against previously untreated neoplasms, not drug-resistant lines.

Therapeutic potentiation may result from
(a) additive antitumor activity without additive host toxicity,
(b) prevention of or delay in the emergence of resistance,
(c) biochemical potentiation with some selectivity for neoplastic cells,
(d) differences in the tissue distribution of drugs of a combination.

* A line of plasmacytoma (Pla-1) selected for resistance to BCNU was cross resistant to cyclophosphamide and TEM; whereas a line of L1210 leukemia selected for resistance to BCNU was sensitive to cytoxan.

FIGURE 22.—Quick reference chart on cross resistance between anticancer agents [*from* ref 56].

cross-resistance characteristics of new compounds, and will provide the clinical investigator with important information pertaining to the sequence of drug administration. In one experimental study, for example, Tyrer et al (figs 23 and 24) (57) described cross-resistance characteristics for a subline of leukemia L1210 resistant to BIC. It is of considerable interest that this subline was cross resistant to DTIC. It was also cross resistant to nitrosourea derivatives including BCNU, but markedly sensitive to cyclophosphamide. The suggestion would be that following the origin of resistance to BIC, cyclophosphamide could be a drug of choice for the clinic.

Determination of the collateral sensitivity characteristics of resistant sublines may provide information of interest. In a notable example a subline of leukemia L1210 resistant to 8-azaguanine and cross resistant to 6-mercaptopurine was found to be more sensitive to methotrexate than the original sensitive leukemic line (table 15) (55). In another study (table 16) (58) it was reported that a number of sublines of leukemia L1210 resistant to known antitumor agents showed increased sensitivity to treatment with BCNU. The occurrence of collateral sensitivity, whether attributable to biochemical alteration of the tumor cells or an alteration in their immunogenicity, may provide further guidance in the choice of drug to be used in sequence following the origin of drug resistance in the clinic.

In the testing of a drug against a spectrum of tumors it may be determined that certain types of tumors are more sensitive or more resistant to the drug. If sensitivity correlations can be established between experimental tumor systems and specific clinical disease types this may provide information of considerable importance for choice of drug for the specific tumor under treatment. It is also worthwhile to determine, where an experimental tumor system is sensitive to a drug, the incidence of nonresponders and to investigate the basis for drug failure.

It is important to conduct biochemical, pharmacologic, and immunologic investigations of resistant tumor cell populations. Such information may be

TABLE 15.—Comparison of effectiveness against advanced leukemia L1210 and the 8-azaguanine- and 6-mercaptopurine-resistant variant, L1210-Ag-R [*from* ref 55]

Agent	% Increase in MST over controls at the optimal daily dose	
	L1210	L1210-Ag-R*
8-Azaguanine	100	8
6-MP	69	5
6-MP-riboside	70	0
6-Isopropylthiopurine	60	8
9-Butyl-6-MP	38	10
Thiamiprine	78	8
Cyclophosphamide	145	112
Methotrexate	159	388

*L1210-Ag-R was developed by Law et al by 8-azaguanine treatment of DBA/2 mice with ascitic leukemia L1210.

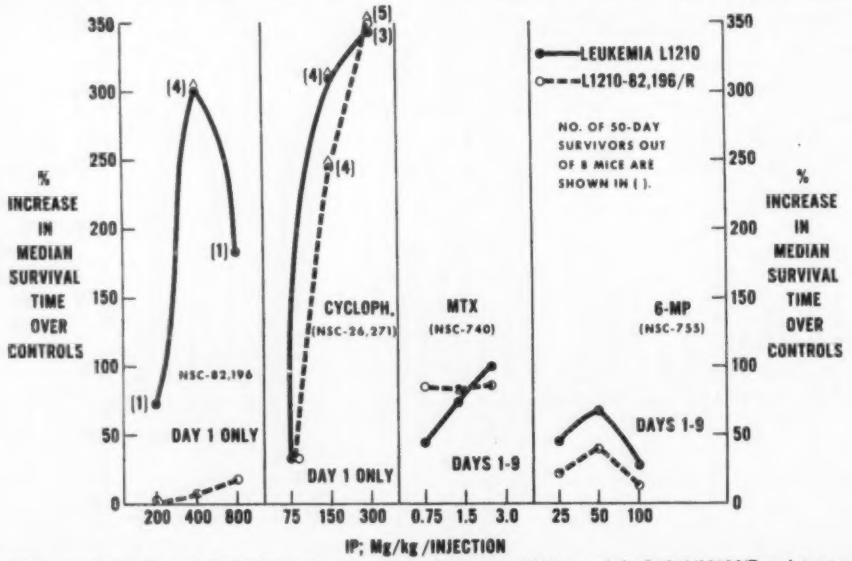


FIGURE 23.—Dose-response plot; effect of antileukemic agents against the parental L1210 and the L1210/82196/R resistant variant. The logarithmically spaced doses are expressed as mg/kg/injection; BDF_1 male mice (22-30 g) were inoculated ip with either ascites parental L1210 (1×10^5 cells) or ascites L1210/82196/R (1×10^5 cells). Eight leukemic mice were treated at each dose level. MST for the 16 untreated controls inoculated with parental L1210 or with L1210/82196/R variant was 9 and 11 days respectively. Cycloph = cyclophosphamide; MTX = methotrexate [*from* ref 57].

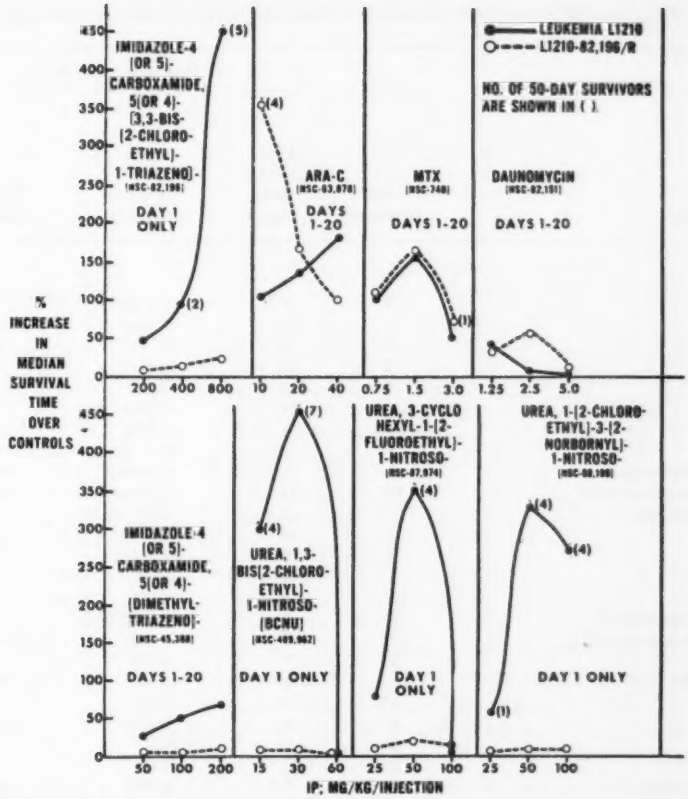


FIGURE 24.—Dose-response plot; effect of nitrosoureas and other antileukemic agents against the parental L1210 and the L1210/82196/R resistant variant. The logarithmically spaced doses are expressed as mg/kg/injection. BDF_1 male mice (21-29 g) were inoculated ip with either ascites parental L1210 (1×10^5 cells) or ascites L1210/82196/R (1×10^5 cells). Eight leukemic mice were treated at each dose level. MST for the 16 untreated controls inoculated with parental L1210 or with L1210/82196/R variant was 9 and 10 days respectively. Ara-C = cytosine arabinoside; MTX = methotrexate [*from* ref 57].

useful in seeking means for prevention of tumor cell resistance and for instituting effective therapy once resistance has occurred.

The utilization of combination chemotherapy in relation to the phenomenon of resistance is described below.

G. Metastasis

Drugs can be characterized for biologic and therapeutic activity in relation to tumor cell dissemination and metastatic disease.

In the screening systems for initial detection of active antitumor agents treatment is generally administered against early stages of the disease at a time when tumor cells are essentially localized. It is important, once it has been determined that a drug is active in the initial screen, to focus attention on the more difficult therapeutic challenge represented by the disseminated tumor. As discussed above, drugs that are highly effective when a low number of tumor cells are present may show markedly diminished effectiveness against advanced stages of disseminated disease (33,44,45,59). The advanced leukemia L1210 system in which the disease is systemic and death is expected within a few days also provides a useful model for this purpose. When the leukemia L1210 cells are inoculated either sc or im, the tumor grows progressively at the local inoculum site and the leukemic cells disseminate systemically within a few days. With treatment against advanced leukemia L1210, measurements can be made of the rate of growth of the local tumor and the lifespan of the animals. In addition, the various tissues of the mouse can be bioassayed to estimate the number of leukemic cells present. The advanced leukemia L1210 system where the leukemic cells are inoculated either sc, im, or ip has been used extensively in nu-

TABLE 16.—MST of mice bearing sensitive or drug-resistant L1210 sublines* [*from* ref 58]

L1210 resistant sublines†	MST of mice receiving—				
	No treatment	Cyclophosphamide Day -1	Drug‡ treatment Days 1-10 (mg/kg)	BCNU Day 6	Cyclophosphamide Day -1, BCNU Day 6
L1210 sensitive	8	8		9	9
6-MP(15)	10	10.5	11.5 (50)	20.5	15.5
6-MP(41)	12	10	10 (50)	>60	12
NSC-39073(57)	10	9	12 (5)	15.5	10.5
Vincristine	13	11	12 (0.2)	14	12.5
Cyclophosphamide	9	9	9 (25)	14	14
Cyclophosphamide (21)	11	10	13 (25)	16	12
Cyclophosphamide (26)	10	11	11 (25)	>60	28
5-Fluorouracil (29)	9	9	13.5 (15)	30	17
Cytosine arabinoside/methotrexate	11	10	11 (5-0.5)	>60	18.5
Cytosine arabinoside/methotrexate (18)	11	11	12 (5-0.5)	>60	22
6-MP/5-fluorouracil/methotrexate	10.5	10.5	14 (20-5-0.5)	18.5	18
cl-TIC	10	10	11.5 (10)	>60	12
TIC	9	9	13.5 (10)	16	14
Methyl-GAG	9	9	10 (30)	19	18
Methyl-GAG	9	9	14 (30)	17	16
Methyl-GAG/cyclophosphamide/6-MP	9	9	11 (60/25/50)	17	15
Isoquinaldehyde, thiosemicarbazone(41)	11	11	11 (40)	41	19
Cytosine arabinoside (30)	9	8	9 (10)	12	10
Cytosine arabinoside (133)	10.5	10	10 (10)	>60	23.5
Methotrexate	8.5	9	12 (1)	18	16
Methotrexate	8	8	15.5 (1)	18.5	16
Methotrexate (14)	9	9	10 (1)	17	17
Methotrexate	9	9	10 (1)	45	20
3′, 5′-Dichloromethotrexate (26)	9	7.5	8 (80)	21	9
3′-Bromo-5′-chloromethotrexate	10	10.5	10 (80)	35	22
BIC	10	9	10 (70)	13	11
NSC-114924 (8)	13	11	10 (25)	24	18
DTIC	15	15	13 (25)	27	18.5
NSC-407347	12.5	12	12 (25)	>60	30
NSC-1402	11	10	10 (40)	>60	24

*CDF_1 female mice were given ip injections of 10^5 leukemic cells on Day 0. Cyclophosphamide = 180 mg/kg ip; BCNU = 18 mg/kg sc.
†Nos. in parentheses are number of treated transplant generations.
‡Treatment with the drug to which the leukemia became resistant.

merous investigations and currently is being applied to the further characterization of various new drugs. An example of a typical experiment, in which treatment was initiated only several days prior to the expected time of death of the animals, is shown in table 17 (14,60).

An initial demonstration of the difficulty of treating more advanced disseminated disease involved the treatment of leukemia L1210 with aminopterin (table 18) (59,61). A single treatment administered up to 6 days after leukemic inoculation was effective in increasing survival time. However, treatment at 9 days, when the disease was highly disseminated, was essentially ineffective. As noted above, treatment with cytosine arabinoside q3h/24h/q4d ("around the clock" treatment) was a highly effective schedule against early leukemia L1210. However, against advanced disseminated disease there was a marked reduction in therapeutic effectiveness with this drug and a loss of schedule dependency (table 6) (33).

Drugs such as guanazole, 5-azacytidine, 5-hydroxypicolinaldehyde thiosemicarbazone, and others, although quite effective against early leukemia L1210, showed reduced effectiveness against advanced systemic disease (table 19) (59).

It is also more difficult to obtain total eradication of leukemic cells when the tumor is disseminated. As discussed earlier, over a series of schedules cytosine

TABLE 17.—Effectiveness of purine derivatives against advanced leukemia L1210* [*from* Ref 14 and 60]

Compound	Daily dose (mg/kg)	MST (days)	Mortality distribution (days after tumor inoculation)																										
			9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Methotrexate	3.02	18.0		1		1			1	2				1				1		1						1				1
	1.81	21.5						2	1		1			1			1					2						1	1	
	1.09	25.5															2	1	2	2	1		1						1
	0.65	24.0															4	2			1	1	1	1						
	0.39	23.5					3			1							1	1		2					1	1				
6-MP	300	16.0							1	5	1	3																		
	180	19.0									1	2	4	2			1													
	108	19.0			1							2	3	1	3															
	64.8	15.5				1	3		1	2					1	1	1													
	38.9	14.0			1	2	1	3	2			1																		
6-Chloropurine	833	10.0		10																										
	500	10.0		10																										
	300	18.0			3					1	1		1	3	1															
	180	12.5		1	3	1	2			2	1																			
	108	17.0			2	1				2		1	3		1															
	64.8	12.0		1	2	3	1	2			1																			
6-MP riboside	500	19.0										3	3	3		1														
	300	19.0									1	1	4	3	1															
	180	20.0										2	1	4	3															
	108	16.5		1	1		92	1					1		3	1														
	64.8	18.0			1		1		1	1	1		1	1	2					1										
	38.9	12.5			4	1	3	1	1																					
	23.3	12.0		2	1	4	3																							
6-(Methylthio)purine, trihydrate	300	10.0		10																										
	180	10.0		6	2						1										1									
	108	14.0		1	1		2	3	2	1																				
	64.8	12.0			3	4	1	2																						
	38.9	12.0		1	2	3	3				1																			
6-Azathymine	300	12.0		1	1	7	1																							
	180	12.0			4	2	3		1																					
	108	13.0		2		2	4	2																						
	64.8	11.5		1	4	3	2																							
	38.9	12.0			3	5	1	1																						
	23.3	12.0		1	1	6	1	1																						
Controls (exp inoc)	—	13.0			2	2	3	3																						
Controls (exp inoc)	—	12.0			3	3	2	1	1																					
Controls (1/10 exp inoc)	—	—																						(6 survivors Day 37)						
Controls (1/100 exp inoc)	—	—																		1				(5 survivors Day 37)						
Controls (1/1000 exp inoc)	—	—																						(6 survivors Day 37)						
Retransplant recipients	—	8.5 (from Day 9)					1				2	1	1						1											
Untreated without tumor	—	—																						(10 survivors Day 37)						

*Mice: BDF_1 female, 19-26 g. Experimental inoculum: 290,000 cells/mouse. Daily treatment begun on Day 9.

TABLE 18.—Effect of day of administration of aminopterin on the survival time of mice inoculated with leukemia L1210 [*from* refs 59 and 61]

Day of drug treatment	Average survival time of mice dying of leukemia (at 50% toxic mortality level): days following leukemic inoculation
1	17.0
3	19.0
6	17.5
9	15
Control	14

*Based on fitted least-squares relationship between time to death from tumor for mice surviving aminopterin toxicity and the calculated probit of toxic mortality from aminopterin.

arabinoside showed a definite loss in ability to cure mice with leukemia L1210 when the disease was more advanced (fig 21) (45).

Advanced solid tumors that have metastasized from the local site of inoculation could provide useful models for the study of new drugs. The Lewis lung carcinoma provides an interesting model for the evaluation of drugs. In one system a tumor cell suspension is injected iv and the animals are then subjected to therapy. After therapy the animals may be killed, the number of lung colonies may be counted, and observations can be made of the pathology of the lung. The influence of therapy on the survival time of the animals may also be determined. In these studies treatment may be initiated shortly after iv inoculation of the tumor cells or at various stages during the development of the disease. The influence of schedules of drug administration can be examined, including optimal schedules as determined from earlier experiments. The drug may be administered by different routes including ip, sc, oral, and iv. An experimental study employing this methodology in the treatment of Lewis lung carcinoma is illustrated in table 20 (36).

Another well-developed model employing the Lewis lung carcinoma involves inoculation of the tumor cells sc or im. Under such circumstance the tumor cells metastasize preferentially to the lungs. In this system treatment can be administered early following tumor inoculation, at a time prior to metastasis to the lungs, or at later stages of the disease at which time there is a large local tumor and extensive metastatic growth in the lungs. Thus, with this system observations can be made on the influence of a drug on the growth of the local tumor, on the metastatic process, and against metastatic disease once it has occurred. For the advanced tumor, observations can be made on the influence of drug both on the local tumor and on the disease in the lung. Parameters of response may include survival time of the animals, bioassay of numbers of viable tumor cells, and pathologic examination of the tissues. A study by Karrer et al (fig 25 and table 21) (62) illustrates the experimental model. Treatment with cyclophosphamide was therapeutically more effective against early disease and diminished in effectiveness following metastasis to the lungs. Surgery plus chemotherapy appeared to be more effective than surgery alone, and this is discussed further below.

There should be investigation of other lung tumor models and novel approaches in relation to clinical disease.

TABLE 19.—Effectiveness of agents against early and advanced leukemia L1210 [*from* ref 59]

Agent	Early L1210*		Advanced L1210†	
	Treatment schedule	%ILS	Treatment schedule	%ILS
Guanazole	q3h, Days 1,5,9	109	q3h, Days 5,9,13	40
			Daily, Days 5-13	50
5-Azacytidine	Days 1,5,9	>200‡	Days 5,9,13	30
			Daily, Days 5-13	40
5-Hydroxypicolinaldehyde thiosemicarbazone	q3h, Days 1,5,9	>400	q3h, Days 5,9,13	75
			Daily, Days 5-13	60
Isophosphamide	Day 1 only	>355	Day 5 only	121
			Days 5,9,13	211
Camptothecin	Days 1,5,9	140‡	Days 5,9,13	89

*Ip tumor, 10^5 ascites, ip treatment.
†Sc tumor, 10^6 ascites, sc treatment.
‡DR&DP screening data.

TABLE 20.—Influence of route of tumor inoculation and route of drug administration on the antitumor activity of adriamycin against Lewis lung carcinoma [*from* ref 36]

Tumor site *	Adriamycin dose (mg/kg/injection)	%ILS			
		Treatment Day 1		Treatment Day 7	
		iv	ip	iv	ip
iv	20	toxic	toxic	toxic	toxic
	14	>300	toxic	60	17
	9.8	90	45	26	21
	6.9	36	17	45	21
	4.8	21	7	12	2
	3.4	—	—	—	—
sc	20	—	—	—	—
	14	toxic	toxic	toxic	toxic
	9.8	toxic	toxic	6	0
	6.9	14	17	1	14
	4.8	0	0	0	0
	3.4	0	1	6	3

*10^5 viable Lewis lung carcinoma cells implanted on Day 0. MST of untreated controls: iv tumor, 21.0 days; sc tumor, 32.0 days.

TABLE 21.—Bioassay of the lungs of mice with Lewis lung tumor [*from* ref 62]

Day of bioassay	Donor mice		Recipient mice	
	Cyclophosphamide		Tumor mortality	
	Day treated	Dose (mg/kg)	No. of animals dead/total treated	Median day of death (range)
8	—	0	4/7	34 (22-45)
8	6	300	0/7	—
8	6	225	0/7	—
8	6	168	0/7	—
8	6	122	0/7	—
15	—	0	11/14	22 (19-44)
15	13	300	0/7	—
15	13	225	1/7	43
15	13	168	2/5	42,47
15	13	122	1/7	47
15	10	300	4/7	21 (19-45)
15	10	225	5/7	20 (17-40)
15	10	168	6/7	25 (19-50)
15	10	122	6/7	22 (18-40)

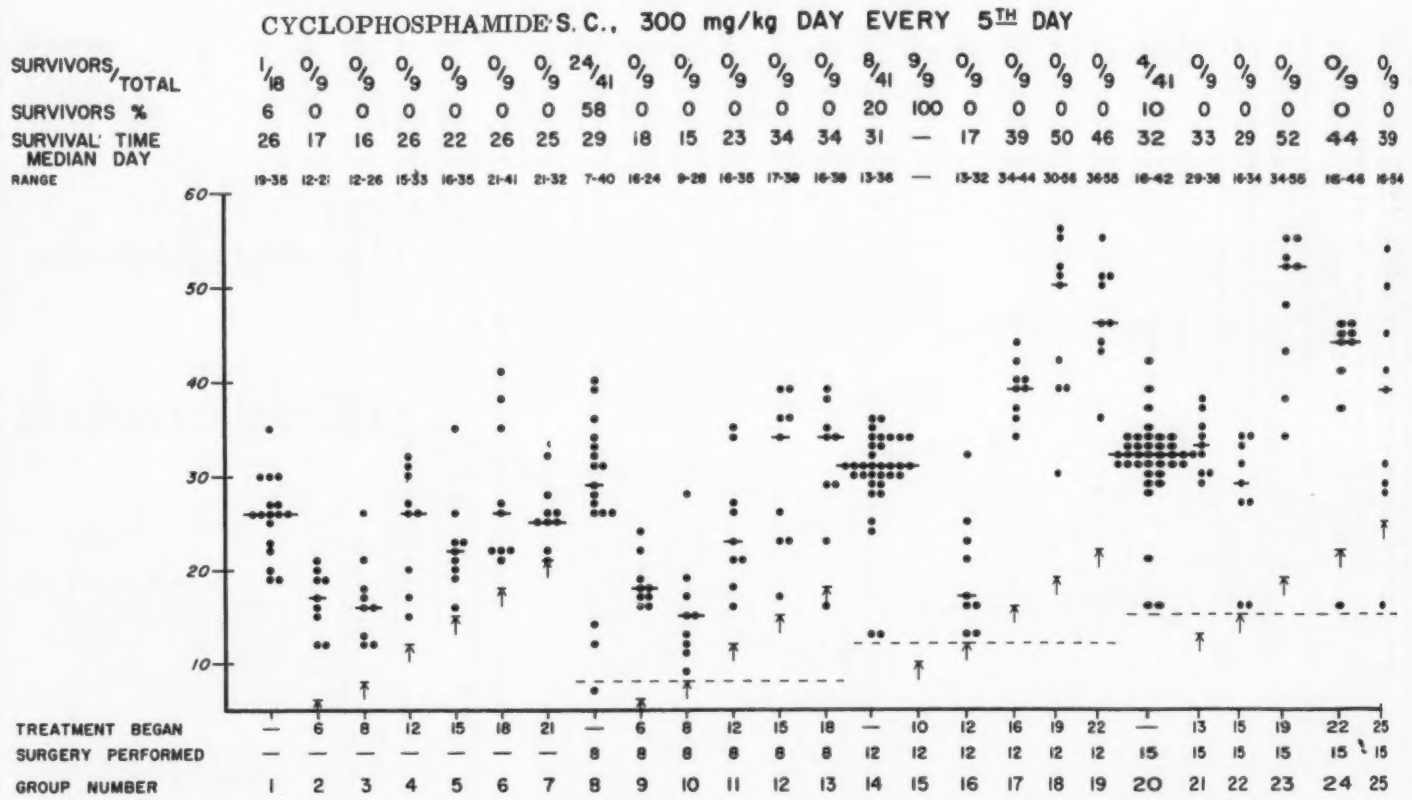


FIGURE 25.—Comparison of the effectiveness of chemotherapy alone, radical surgical removal of the primary implanted tumor alone, and surgery plus adjuvant chemotherapy on the survival time and number of survivors of mice inoculated with Lewis lung tumor. The survival time of the dead animals is indicated on the ordinate in days. The number of survivors is determined at the end of the experiments on Day 62. Treatment was with 300 mg/kg cyclophosphamide administered sc at various times from Days 6 to 25 after tumor inoculation. In groups receiving surgery (on Days 8, 12, or 15) plus adjuvant chemotherapy, drug treatment was initiated either before surgery, at the time of surgery, or at various times following surgery. On Day 8 the mice had visible evidence of local tumor growth (6-8 mm average diameter). By Day 15 the average tumor diameter was 12-20 mm [*from* ref 62].

It is important to provide models to determine if treatment prevents metastasis to sequestered sites. In one study referred to above (52) a model was employed by means of which it could be determined whether treatment with a drug that prolongs survival time of mice with leukemia L1210 permits infiltration of the tumor cells to the brain.

Hellmann et al (63) have observed that treatment of Lewis lung carcinoma with ICRF-159 tended to prevent metastasis from a primary tumor site by maintenance of the integrity of capillary structure in association with the tumor. The influence of new drugs on the invasiveness, anatomy, and physiology of the tumor vasculature would provide information of value.

Any immunosuppressant action of a drug may have an important effect on the origin of metastases, and models should be developed for the estimation of immunosuppressant action on the metastatic process. Treatment of naturally resistant or induced-resistant tumors with an immunosuppressant drug could result in increased rate of tumor growth and metastasis, complicating clinical application. This is discussed further below.

Models may also be applied to answer questions such as: Does pretreatment or posttreatment with the drug destroy existing metastases or prevent metastasis from occurring as the result of surgery? Where treatment has been effective, how long does the metastasis-free state continue after discontinuation of therapy? Models could be devised that parallel the clinical course and status of disease. For example, a breast tumor model with nodal infiltration would be desirable as a dissemination model for drug treatment as well as for treatment with combined modalities. The utilization of appropriate models for the investigation of new drugs may lead to the more effective treatment of advanced disseminated disease.

H. Drug Action on Immune Response of Host

The extent of immunosuppression of the host by a drug may influence its therapeutic action. A series of relatively standardized tests can be employed to measure immunosuppressive action on both cellular and humoral responses of the host and examples may be cited.

Effect of drugs on cell-mediated immunity

The mice may be immunized with irradiated allogeneic tumor cells. They may be treated with the test drug (before, during, or after immunization) and the lymphocytes may subsequently be removed (attacker cells) and reacted against target cells of the same tumor in vitro. One method involves release of chromium 51 from labeled target cells as a measure of cytotoxicity.

Effect of drugs on host-versus-graft reactions

The extent of inhibition by the drug of the allograft response in vivo can be determined. In one system reported by Humphreys et al (64) (table 22) allogeneic C57BL mice were pretreated with the test drug and leukemia L1210 (antifolic resistant) was inoculated when it was estimated that the drug had been cleared from the host. The percentage of leukemia "takes" was measured under such circumstances. The higher the percentage of "takes" the greater the degree of immunosuppression.

Another model for measuring the influence of a drug on the allograft reaction involves the treatment of a tumor resistant to the test drug in a host with histocompatibility differences. For example leukemia L1210 of DBA/2 origin, when transplanted sc in BALB/c mice at an appropriate inoculum level, will grow initially and then regress. If the drug is immunosuppressive the tumor will grow progressively until the death of the host (table 23) (65).

In another system the mice may be pre-immunized with non-viable tumor, carrying tumor-associated transplantation antigen(s). The immunized host is treated with the drug and rechallenged. Immunosuppression is reflected in the extent of growth of the rechallenge inoculum.

A peritoneal inhibition test can be conducted. In this system the mice are sensitized with incompatible tumor or allogeneic lymphocytes. Following this the same tumor cells are injected ip and radiolabeled ^{125}IUdR is injected. The cells are removed after 24 hours and the extent of radioactivity is measured. Immunosuppression permits tumor cell proliferation and this is reflected in higher incorporation of ^{125}IUdR (66).

The influence of the drug in permitting chimera formation may serve as a measure of its immunosuppressant activity. If transferred hemi-allogeneic cells persist in the host following drug therapy, as reflected for example in persistence of allogeneic gamma globulin, then chimera formation has been successful and the drug is immunosuppressive (67).

The influence of the drug on prolongation of skin allograft survival can also be measured.

Effect of drugs on graft-versus-host (GVH) reactions

The ability of the drug to block the GVH reaction in vivo can be determined. In the assay developed by Simonsen and Jensen (68), newborn mice are injected with allogeneic lymphoid cells and the spleen weight

TABLE 22.—Effects of various agents on the percentage take of FR-3 (an antifolic-resistant subline of leukemia L1210) in homologous C57BL mice [*from* ref 64]

Drug	Dosage* (mg/kg/day)	Average weight loss† (g)	No. of toxic deaths/ total treated	Tumor takes(deaths)/ total at inoculum concentration (%) of —		
				10‡	1.0	0.1
Methotrexate	9.4	4.6	5/9	1(0)/2§	0/1	0/1
	7.1	2.0	1/9	0/2	0/3	0/3
	5.3	—	—	0/3	0/3	0/3
6-MP¶	94	5.8	3/9	3(1)/3	0/1	0/2
	71	4.2	2/9	1/2	0/3	0/2
Cyclophosphamide	168	6.7	6/9	1(0)/1	—	1(1)/2
	126	2.0	—	3(0)/3	2(0)/3	1(0)/3
	94	2.0	—	3(0)/3	2(0)/3	1(0)/3
Triethylenemelamine	1.68	7.0	3/9	2(0)/2	2(0)/2	2(0)/2
	1.26	3.3	—	3(0)/3	3(0)/3	3(0)/3
Melphalan¶	0.94	1.9	—	2(0)/3	3(0)/3	2(0)/3
	9.4	7.1	2/9	3(3)/3	2(2)/2	2(2)/2
	7.1	3.8	—	3(3)/3	3(1)/3	3(0)/3
X-radiation	168 rads	4.3	—	3(3)/3	2(2)/3	1(1)/3
	126 rads	3.2	—	3(3)/3	2(1)/3	0/3
Homologous (C57BL) controls			—	0/9	0/9	0/9
Isologous (CDBA) controls			—	3(3)/3	3(3)/3	3(3)/3

*Drugs and irradiation administered for 5 consecutive daily treatments.

†Average maximum weight loss for each animal.

‡10% inoculum = 1 spleen in 10 ml normal saline. Each mouse received 0.1 ml sc in right inguinal region 24 hrs after the last treatment with either drug or radiation therapy.

§One take and no progressive tumor growth was observed in 2 mice.

¶Animals treated with 126 mg/kg 6-MP, 12.6 mg/kg melphalan, and 225 rads of radiation exhibited acute toxicity and marked weight loss. All animals in these groups died due to toxicity prior to growth of the tumor in isologous controls.

is determined after 10 days. With this system test drug can be employed either to treat the donor of allogeneic lymphocytes or to posttreat the recipient. If the drug is immunosuppressive for the donor, there will be a diminished increase in the weight of the spleen. Posttreatment, if effective, will kill proliferating cells and there will be little or no increase in spleen weight.

In another system adult mice can be lethally irradiated followed by injection of allogeneic bone marrow cells (69). The ^{125}IUdR uptake of the spleen is measured 5 days later, before death. If the donor is treated with an immunosuppressive drug its bone marrow cells fail to proliferate in the recipient and ^{125}IUdR uptake will be reduced. If the irradiated recipient is treated after bone marrow cell transfer, again there may be inhibition of ^{125}IUdR uptake.

Effect of drugs on lymphocyte proliferative response in vitro

The action of the drug on stimulation of DNA synthesis in vitro by nonspecific mitogens may be determined (70). These mitogens include phytohemagglutinin (PHA), concanavalin A, pokeweed mitogen, and *Escherichia coli* lipopolysaccharide (LPS). The drug could be tested in vivo by treating the animal prior to removal of lymphocytes or in vitro by adding the drug to the lymphocyte culture.

In the mixed lymphocyte culture method (71) allogeneic lymphocytes from two strains of mice are cocultured. For example, DNA synthesis and multiplication of spleen cells of C57BL mice may be blocked in vitro by treatment with mitomycin C. The C57BL cells treated with mitomycin C are then mixed with viable DBA/2 spleen cells. In this system, although the C57BL lymphocytes treated with mitomycin C do not divide, they still retain alloantigens which act as specific stimulants of DNA synthesis for the DBA/2 cells. The cells are cultured for 3 or 4 days, after which tritiated thymidine is added and the extent of incorporation of tritiated thymidine into DNA is measured as a reflection of the extent of DNA synthesis. Drug may be added in vitro or the DBA/2 donor animals may be treated. If the drug is immunosuppressive, there is no stimulatory response.

TABLE 23.—Effect of treatment with methotrexate on homograft response and on immune response in reinoculated mice: sensitive L1210Z leukemia and the methotrexate-resistant subline M46R* [*from* ref 65]

			First period (Days 0-20)							Second period (Days 21-41)						
				Tumor occurrence				Survivors			Tumor occurrence				Survivors	
Group	Methotrexate dose (mg/kg)	Schedule (daily)	Inoculum† (Day 0)	No. of takes/ total	Average maximum tumor diameter: mm	Average maximum tumor diameter: Day	Tumor duration: 2 longest (days)	No. of survivors/ total	Median day of death	Inoculum‡ (Day 21)	No. of takes/ total	Average maximum tumor diameter: mm	Average maximum tumor diameter: Day	Tumor duration: 2 longest (days)	No. of survivors/ total	Median day of death
			Tumor growth and regression in incompatible host													
1	0		L1210Z	7/9	10.9	9.7	15	9/9							9/9	
2	0		M46R	9/9	12.3	9.4	17,20	5/9	10						5/5	
3	0							9/9		L1210Z	9/9	10.8	8.4	15	6/9	9
4	0							9/9		M46R	9/9	11.4	9.4	20, >20	7/9	12.5
			Inhibition by drug of sensitive tumor in incompatible host													
5	1.0	0-41	L1210Z	0/9				9/9							9/9	
6	1.0	21-41						9/9		L1210Z	0/9				9/9	
			Lethality with drug treatment of resistant tumor in incompatible host													
7	1.0	0-20	M46R	9/9	11.8	8.4	>11	0/9	11						0/0	
8	1.0	21-41						9/9		M46R	9/9	11.3	10.7	>14, >16	1/9	12
			Tumor growth and regression							*Immunity of incompatible host to reinoculation*						
9			L1210Z	8/9	10.4	9.8	17	9/9		L1210	0/9				9/9	
10			L1210Z	7/9	11.7	9.1	15, 17	9/9		M46R	0/9				9/9	
11			M46R	9/9	13.0	8.9	15	6/9	9	M46R	0/6				6/6	
			Tumor growth and regression							*Retained immunity with drug treatment*						
12	1.0	21-41	L1210Z	9/9	10.4	9.7	15	9/9		L1210Z	0/9				9/9	
13	1.0	21-41	L1210Z	9/9	10.1	8.7	15, 17	9/9		M46R	0/9				9/9	
14	1.0	21-41	M46R	9/9	13.3	11.0	17, >20	7/9	13	M46R	0/7				6/7§	4
			Nontumorous control													
15	1.0	0-41						9/9							9/9	
16								5/5							5/5	

*BALB/cAn male mice.

†One spleen plus 9 ml normal saline for both leukemias. The inoculum concentration for each leukemia was 100 times greater than that required to give 100% takes in control CDBA hybrid male mice.

‡One spleen plus 9 ml normal saline for both leukemias. The inoculum concentration for each leukemia was 1000 times greater than that required to give 100% takes in control CDBA hybrid male mice.

§This group was inadvertently treated with methotrexate on Day 8. In 1 mouse the tumor eventually attained a diameter of 18 mm (in the second period) and the mouse died on Day 25 (Day 4 for the second period).

Effect of drugs on humoral immune responses

The primary plaque-forming cell response can be utilized (72). In one system sheep red blood cells (SRBC) are injected into the host after which the spleen cells of the host are removed and reacted against SRBC in agar suspension. 19S antibody can be measured 2-4 days after treatment, at which time it is predominant. 7S antibody reaches its peak at later times. Thus the spleen may be removed at various times following injection of SRBC to follow the kinetics of the immunoresponse. In this system the drug can be administered before, after, or concomitantly with the antigen and the analysis can be conducted at different times.

The secondary plaque-forming cell response can also be measured (72). This system measures predominantly 7S antibody. In this system the animals are immunized with SRBC and then subsequently given a booster injection with the same antigen. The drug being tested is administered either before or after either injection of SRBC. An immunosuppressive drug will reduce the number of plaque-forming cells.

It will be most useful to subject new drugs to testing for immunosuppressive activity in systems that will provide information of greatest relevancy to clinical therapy.

I. Combination Chemotherapy

There is extensive clinical interest in the use of polychemotherapy for clinical neoplasia (73-78). Characterization of a new drug, employed in combination with one or more of the known antitumor agents, is highly desirable and may be helpful in the determination of appropriate combinations for clinical utilization. The studies may provide additional important information concerning dosages, drug ratios, schedules, routes, and so forth for the combinations.

A metabolite plus an antimetabolite may be employed in combination chemotherapy with a view to obtaining differential protection for the host by the metabolite with retention of the antitumor toxicity of the antimetabolite. Such an advantage in therapy has been obtained experimentally employing the combination of citrovorum factor plus either aminopterin or methotrexate. The experimental design employed to determine the effect of delayed administration of citrovorum factor on the response to aminopterin of mice with leukemia L1210 is illustrated in table 24 (79), and summary data for this experiment are presented in figure 26 (79). When the citrovorum factor was administered concomitantly with the aminopterin there was proportionately greater protection for the leukemic cells than for the host by the citrovorum factor and there was a net loss in therapeutic effectiveness. On the other hand, when administration of citrovorum factor was delayed for 12-24 hours relative to the aminopterin, the action of the aminopterin was essentially complete with respect to destruction of leukemic cells, whereas it was still possible for the citrovorum factor to reverse the host toxicity of the aminopterin. This in turn permitted the employment of higher doses of aminopterin in conjunction with delayed administration of citrovorum factor and a resultant net increase in therapeutic response occurred. Similar results have been obtained with methotrexate plus delayed administration of citrovorum factor (80, 81). This type of methodology, involving methotrexate and delayed administration of citrovorum factor, has been carried over with success to the clinic in the treatment of leukemia and osteogenic sarcoma (82-86). It is suggested that more detailed studies with the antimetabolites currently in clinical use be conducted with their corresponding metabolites in an attempt to determine whether improved therapeutic response can be obtained. It is also suggested that for new antimetabolites, the corresponding metabolites be tested for protective action and also for timing relationships and schedules that may elicit a

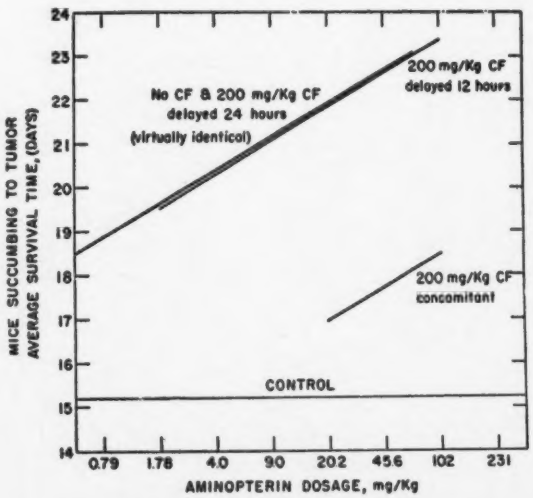


FIGURE 26.—Effect of concomitant and delayed administration of citrovorum factor (CF) on the survival time-aminopterin dosage relationship in leukemic mice. Fitted least squares relationships between time to death from tumor for mice surviving aminopterin toxicity and the logarithm of the dose of aminopterin are plotted. Separate common slope lines are shown for the treatments employed. Lines are drawn to extend over the dosage range at which there were any survivors. The line for aminopterin alone (no CF) extends from 0.52 to 6.00 mg/kg aminopterin. The line for aminopterin with CF administered 24 hrs later extends from 1.18 to 68.4 mg/kg aminopterin [*from* ref 79].

TABLE 24.—Effect of delayed administration of citrovorum factor (CF) on the response of leukemic (L1210) mice to aminopterin (experiment 3) (aminopterin administered 3 days after tumor inoculation = 0 hr)* [*from* ref 79]

Aminopterin (0 hr)	CF† (mg/kg)			No. of drug toxicity deaths/ total treated	Calculated probit of toxic mortality‡	No. of mice surviving drug toxicity and dying from tumor: days after tumor inoculation																
	0 hr	+12 hrs	+24 hrs			14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	...	32
0.52				1/12	3.73			1	5	2	1	1	1									
0.79				1/12	4.09			1	2	4	2	1	1									
1.18				4/12	4.45					2	2	2	1				1					
1.78				3/12	4.81					2	3	2		1	1							
2.67				8/12	5.17					1		2			1							
4.00				11/12	5.53							1										
6.00				7/12	5.89						1		2		2							
9.00				12/12	6.25					(no survivors)												
13.5				12/12	6.61					(no survivors)												
20.2	200			0/10	3.24		3	3	1	2		1										
45.6	200			0/10	3.96			3	4	1	1	1										
102	200			1/10	4.68				1		6	1			1							
231	200			10/10	5.40					(no survivors)												
519	200			10/10	6.12					(no survivors)												
1.78		200		0/12	2.18				3	2	3	2	1						1			
2.67		200		0/12	2.54					4	3	1	2			1				1		
4.00		200		0/12	2.90					2	2	2	4			2						
6.00		200		1/12	3.26					1	2	2	1	3	1	1						
9.00		200		1/12	3.62					2	1	5		2		1						
13.5		200		2/12	3.98						1	5		3					1			
20.2		200		3/12	4.34						2	2	1		1	1	1					1
30.4		200		4/12	4.70						1		3	1	1	1			1			
45.6		200		7/12	5.06									3		1				1		
68.4		200		9/12	5.42								1		1							
102		200		8/12	5.78								1	2	1							§
1.18			200	0/12	2.67				1	3	2	3	2	1								
1.78			200	1/12	3.03				1	1	3	1	3		1		1					
2.67			200	2/12	3.39					1	6	1	2									
4.00			200	4/12	3.75						3	2		2				1				
6.00			200	2/12	4.11					1	1	2	3	2		1						
9.00			200	4/12	4.46							3	3	1								§
13.5			200	3/12	4.82							5	1	1		1		1				
20.2			200	4/12	5.18						1	3	1		1	1						§
30.4			200	9/12	5.54							1		1	1							
45.6			200	8/12	5.90							1		2			1					
68.4			200	11/12	6.26														1			
Controls:																						
2% $NaHCO_3$¶			0.5% $NaHCO_3$	0/12		1	8	1	1	1												
2% $NaHCO_3$			200 mg/kg CF	0/12		3	7	2														
Untreated				0/12			9	2	1													
Extra untreated**				0/17		5	5	7														

*Inoculum: 89,000 cells/mouse.

†Mice receiving no CF were treated with the CF vehicle (0.5% $NaHCO_3$) at +24 hrs.

‡Calculated probits obtained from the fitted maximum likelihood dose-response curve. A 4-way common probit slope curve was fitted.

§One mouse apparently tumor free at 90 days.

¶Aminopterin vehicle.

**Extra mice that remained after the animals were randomized into the various treatment groups.

therapeutic advantage over the drug employed alone.

That the schedule of administration may influence the combination of a metabolite plus an antimetabolite is illustrated in studies with antifolates plus citrovorum factor where the drugs are administered on different schedules. In one study (table 25 and fig 27) (87) it was observed that treatment with aminopterin on Days 2 and 6 accompanied by delayed administration of citrovorum factor was more effective than treatment with aminopterin on Days 2 and 6 without citrovorum factor; it was also more effective than other schedules of aminopterin with delayed citrovorum factor.

The combinations of drugs employed in experimental and clinical studies generally consist of known antitumor agents. Standardized preclinical methodology has been established for experimental models involving two or three drugs, and modified models may be useful in the exploration of activity for four or more drugs.

Consideration of the host-tumor-drug relationship is important not only for drug treatment with individual drugs but also for combination chemotherapy (55,88,89). Accordingly, synergism was defined broadly as the effect of combining agents to provide an improved therapy (55,88,89). The term "therapeutic synergism" was used to describe instances in which drugs in combination produced a therapeutic response superior to the maximum response to either drug alone (55). Most of the experimental studies to date in combination chemotherapy have been conducted with the leukemia L1210 system with survival time of the animals as the primary index of therapeutic response. A typical experimental design in which the combination of methyl-GAG and 6-MP was investigated is shown in figure 28 (55). It illustrates the importance of the dosage ratio in determining the effectiveness of the drugs in combination and shows the therapeutic synergistic effectiveness against advanced mouse leukemia L1210. A series of dosages is tested for the drugs individually and in combination. For the combination of methyl-GAG plus 6-MP a number of dosage ratios ranging from 4:1 to 1:4 (in mg/kg) elicited a response greater than the maximum response observed for either drug alone. The optimal dosage level when the drugs were combined in a methyl-GAG:6-MP ratio of 1:2 on an mg/kg basis produced the greatest extension in survival time. The optimal combination treatment included each drug at one half its optimal dose.

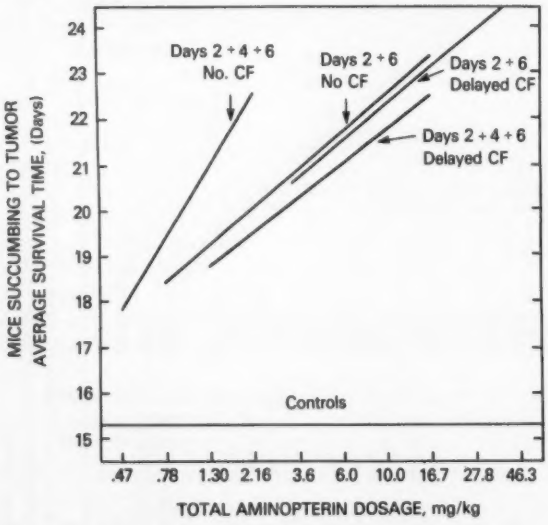


FIGURE 27.—Effect of multiple treatment schedules of aminopterin, with and without citrovorum factor (CF), on the survival time-aminopterin dosage relationship in leukemic mice. Fitted least squares relationships between time to death from tumor for mice surviving aminopterin toxicity and the logarithm of the dose of aminopterin are plotted. For treatment on Days 2 and 6 there was no significant difference in slope for the no CF and delayed CF lines and these are shown as parallel. For treatment on Days 2, 4, and 6, the no CF and delayed CF lines had significantly different slopes and are shown here as nonparallel. Lines are drawn to extend over the dosage range at which there were any survivors [from ref 87].

The drug combinations may be selected on an empirical basis or on the basis of a biochemical rationale. One experiment in which two active drugs were employed in combination is shown in figure 29 (90). It illustrates that the combination of methotrexate plus BCNU administered daily was more effective than methotrexate alone or BCNU alone in the treatment of advanced leukemia L1210.

Methotrexate plus 5-fluorouracil was more effective than methotrexate alone or 5-fluorouracil alone in increasing the survival time of mice with leukemia L1210 (table 26 and fig 30) (91). Both drugs contribute to the inhibition of DNA synthesis, methotrexate providing a block of dihydrofolate reductase (92) and 5-fluorouracil, as the deoxyribotide, resulting in inhibition of thymidylate synthetase (93,94). In another study treatment with hydroxyurea plus cytosine arabinoside was shown to be more effective than hydroxyurea alone or cytosine arabinoside alone in extending the survival time of mice with leukemia L1210 (table 27) (95,96). The use of these two drugs represents a form of sequential biochemical blockade of enzymes involved in DNA synthesis since hydroxyurea is an inhibitor of ribonucleotide reductase (97) and cytosine arabinoside is an inhibitor of DNA polymerase (98).

Additional instances in which therapeutic synergism occurred in the treatment of leukemia L1210 were with the combinations of cytosine arabinoside plus 6-thioguanine (96,99), ICRF-159 plus daunoru-

TABLE 25.—Host and tumor response to aminopterin with and without delayed administration of citrovorum factor (CF): single and multiple treatment* [*from ref* 87]

Total aminopterin dose (mg/kg)	Day of aminopterin administration											
	Day 4			Day 2 (CF Day 2.5)			Day 4 (CF Day 4.5)			Day 6 (CF Day 6.5)		
	D_1	P	ST/D_2	D_1	P	ST/D_2	D_1	P	ST/D_2	D_1	P	ST/D_2
0.78	0	3.32	18.2/10									
2.16	1	4.25	19.4/9									
3.60	6	4.71	20.3/4									
6.00	4	5.17	20.0/6	0	3.35	18.3/10	0	3.47	19.7/10	2	3.04	18.1/8
10.0	7	5.63	23.0/3	1	3.82	18.4/9	2	3.93	20.1/8	2	3.50	18.6/8
16.7	10	6.10		3	4.28	19.6/7	4	4.39	20.2/6	0	3.96	19.3/10
27.8				2	4.74	19.6/8	4	4.85	19.8/6	0	4.42	20.0/10
46.3	10	7.02		8	5.20	20.5/2	7	5.32	22.0/3	3	4.89	19.7/7
77.2				8	5.67	20.0/2	7	5.78	23.0/3	7	5.35	19.3/3
214				9	6.59	21.0/1	9	6.70	22.0/1	10	6.27	

	Days of aminopterin administration											
	Days 2+6			Days 2+6 (CF Days 2.5+6.5)			Days 2+4+6			Days 2+4+6 (CF Days 2.5+4.5+6.5)		
	D_1	P	ST/D_2	D_1	P	ST/D_2	D_1	P	ST/D_2	D_1	P	ST/D_2
0.47							0	2.09	17.6/10			
0.78	0	1.90	18.1/10									
1.30							1	4.20	21.7/9	0	1.02	18.9/10
2.16	2	3.25	19.1/8				6	5.26	21.5/4			
3.60	1	3.92	22.2/9	0	2.35	20.3/10	10	6.32		1	3.14	20.6/9
6.00	4	4.60	22.3/6	0	3.02	21.7/10	10	7.38		3	4.20	20.1/7
10.0	5	5.27	21.8/5	0	3.70	23.1/10	10	8.45		4	5.26	21.7/6
16.7	7	5.94	22.7/3	2	4.37	23.0/8				9	6.31	26.0/1
27.8				6	5.04	23.5/4	10	10.55		10	7.37	
46.3	10	7.29		8	5.72	23.5/2				10	8.43	
77.2				10	6.39							
129										10	10.55	
214				10	7.74							

*Inoculum = 135,000 cells/mouse. D_1 = deaths from drug toxicity. ST/D_2 = average survival time in days for mice surviving drug toxicity but dying of tumor/No. of mice on which this average is based. P = calculated probit of toxic mortality. Calculated probits obtained from the fitted maximum likelihood dose-response curves. A 4-way common probit slope was fitted for groups receiving a single treatment with aminopterin with and without CF. A 2-way common probit slope was fitted for groups receiving aminopterin on Days 2+6 with and without CF. A 2-way common probit slope was fitted for groups receiving aminopterin on Days 2+4+6 with and without CF. CF dose = 200 mg/kg at each interval indicated; day of tumor inoculation = Day 0. Controls: 88 mice in various control groups (2% $NaHCO_3$; untreated) all died of tumor between the 13th and the 18th day following tumor inoculation; average = 15.3 days.

bicin (100), and *N*-acetyl-L-cysteine plus isophosphamide (101). With these combinations it was not necessary to reduce the dosage of the individual drugs when employed in combination, so that the total dosage equivalency for the combination was greater than for the drugs used individually.

New drugs, when employed in combination, can be subjected to schedule-dependency evaluation. For combinations of drugs, as well as for individual drugs, the schedule of administration may determine the extent of therapeutic synergism. In one study with leukemia L1210, treatment with 6-MP and azaserine in combination was tested on three different schedules (fig 31) (102). The combination of 6-MP and azaserine administered every second day was considerably more effective than the drugs employed individually in increasing the survival time of the animals. When the drugs were administered daily, therapeutic synergism was still evident but was less extensive. There was no therapeutic advantage

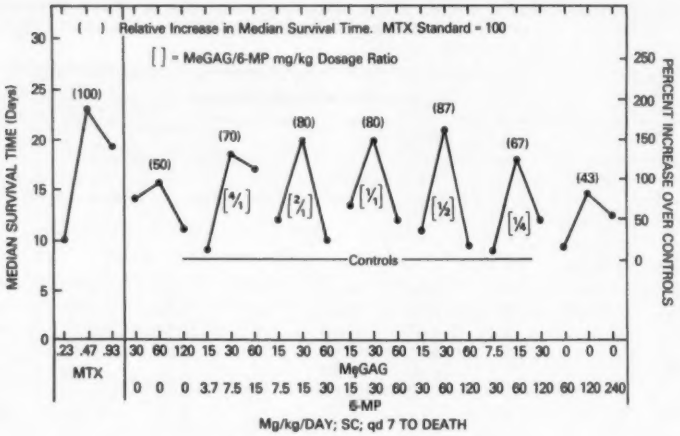

( ) Relative Increase in Median Survival Time. MTX Standard = 100
[ ] = MeGAG/6-MP mg/kg Dosage Ratio
MEDIAN SURVIVAL TIME (Days)
PERCENT INCREASE OVER CONTROLS
(100)
(50)
(70)
(80)
(80)
(87)
(67)
(43)
Controls
.23 .47 .93 30 60 120 15 30 60 15 30 60 15 30 60 15 30 60 7.5 15 30 0 0 0
MTX
MeGAG
0 0 0 3.7 7.5 15 7.5 15 30 15 30 60 30 60 120 30 60 120 60 120 240
6-MP
Mg/kg/DAY; SC; qd 7 TO DEATH

FIGURE 28.—Relative effectiveness of methyl-GAG and 6-MP, separately and in combination, against advanced leukemia L1210. Sc treatment was begun 7 days after sc tumor inoculation and continued daily until death. The dosage levels shown were selected from a wide range of single drug and combination levels. Therapeutic effectiveness is shown in parentheses relative to the effectiveness of the optimal daily dose of methotrexate (MTX) alone employed as a standard [*from* ref 55].

when the drugs were administered in combination twice daily compared with the drugs administered individually.

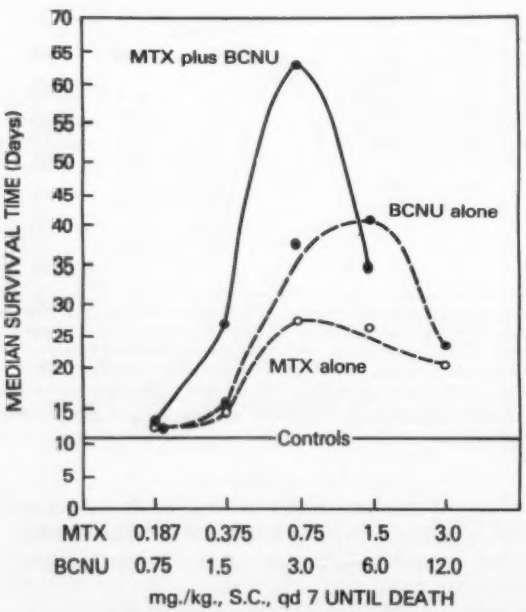


FIGURE 29.—Comparison of methotrexate (MTX) and BCNU, separately and combined, in the daily treatment of advanced leukemia L1210. In this experiment the drugs were combined over a wide range of doses at 5 BCNU:MTX dosage ratios on an mg/kg basis. The combination ratio shown (BCNU:MTX = 4:1) included the maximally effective combination treatment level [*from* ref 90].

The sequence of drug administration may be of considerable importance in determining the therapeutic outcome of combination therapy. Adriamycin is highly effective in the L1210 system as a single treatment but fails to show additional response on multiple treatment. Treatment with adriamycin on a single day followed by daily administration of methotrexate was more effective than adriamycin alone

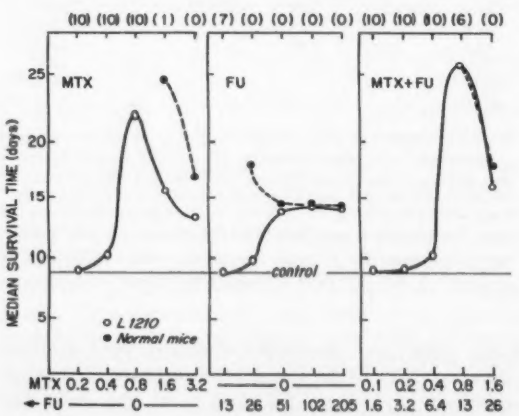


FIGURE 30.—Dose response of normal and leukemic (L1210) mice to treatment with 5-fluorouracil (FU) and methotrexate (MTX), alone and in combination. The doses of the drugs are given in mg/kg and were administered, sc, from the 6th day after leukemic inoculation. Treatment was given daily until death or until the 70th day. Numbers in parentheses = normal animals alive on the 70th day [*from* ref 91].

TABLE 26.—Combination treatment of advanced leukemia L1210 with methotrexate (MTX) plus 5-fluorouracil (FU) in mice* [*from* ref 91]

Experiment No.	Agent	Optimal treatment levels (mg/kg)		Ratio of FU to MTX	Test agent				Controls		Relative increase in MST at optimal dose over controls†	
					Separately		Combined					
		Separately	Combined		Range of individual survival times (days)	MST (days)	Range of individual survival times (days)	MST (days)	Range of individual survival times (days)	MST (days)	Separately	Combined
3	MTX	0.8	1.6	4:1	9-23	17	15-26	21	8-12	10	100	157
	FU	51.2	+6.4		13-16	14					57	
4	MTX	0.5	1.0	8:1	11-28	24.5	27-35	31.5	9-11	10	100	148
	FU	64.0	+8.0		14-15	14					28	
5	MTX	1.08	1.08	10:1	17-22	20	19-30	24	8-9	8	100	133
	FU	50.0	+10.8		13-17	16					67	
6	MTX	0.8	0.8	16:1	10-29	22	18-30	26	8-10	9	100	131
	FU	51.2	+12.8		13-15	14					38	
7	MTX	0.8	1.6	4:1	16-27	23.5	24-26	25	9-14	10	100	111
	FU	51.2	+6.4		14-16	15					37	

*Daily sc treatment was begun 6 or 7 days after sc tumor inoculation; CDBA and BDF_1 male and female mice (18-27 g) were employed; 10 mice in treated groups and 20 mice in untreated group.

†MTX alone daily = 100.

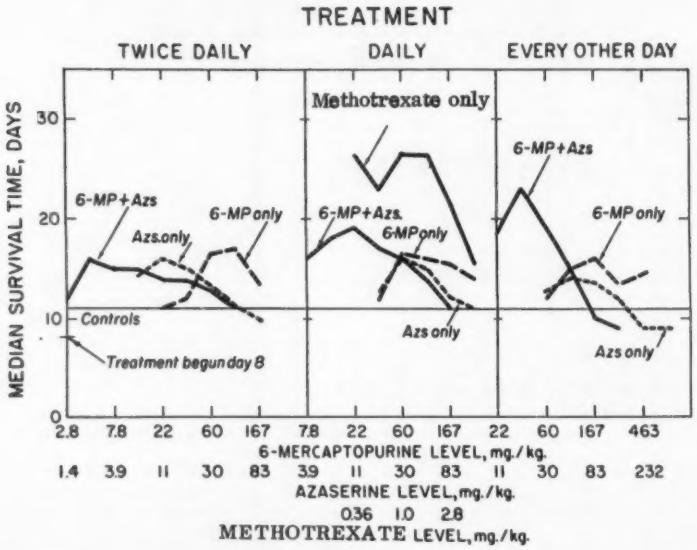


FIGURE 31.—Variation in the therapeutic synergism of 6-MP and azaserine (Azs) for advanced leukemia L1210 with schedule of treatment employed. Daily administration of methotrexate alone is still more effective than any of the synergistic combinations. MSTs shown are based on 8 treated mice per group and 16 untreated controls [*from* ref 102].

or methotrexate alone in increasing the survival time of leukemic mice (table 28) (103). In another experiment involving alteration of sequence it was observed that treatment of leukemia L5178Y with methotrexate followed by L-asparaginase was more effective than the drugs employed individually. On the other hand, treatment with L-asparaginase followed by methotrexate was essentially ineffective (fig 32) (104).

A new drug can be evaluated in combination as well as individually with respect to ability to prevent the origin of tumor cell resistance. In one study employing a combination of cytosine arabinoside plus cyclophosphamide, it was shown that resistance occurred later with the combination than with the individual drugs (table 29) (105).

The new drug may be useful in combination in the treatment of metastatic disease. In one study (table 30) (106) the combination of cyclophosphamide plus methyl-CCNU was more effective in the treatment of advanced Lewis lung disease, where metastasis had occurred to the lungs, than were the drugs individually.

A new drug may be tested for its action on the cell cycle of normal and tumor cells. A new drug can be tested for its potential value in therapy involving

TABLE 27.—Therapeutic synergism in the treatment of leukemia L1210 (ip) with cytosine arabinoside plus hydroxyurea [*from* refs 95 and 96]

Treatment*	Highest non-lethal dose (mg/kg)	ILS (%)	60-Day S/T†
Cytosine arabinoside alone	15	271	0/10
Hydroxyurea alone	60	42	0/10
Cytosine arabinoside + hydroxyurea	10 + 40	>750	6/10

*Treatment ip, q3h × 8, Days 2,6,10,14.
†S/T = survivors/No. treated.

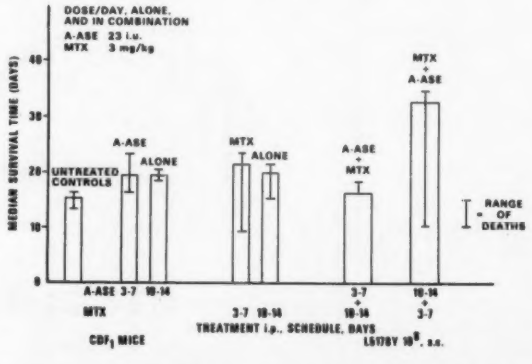


FIGURE 32.—Schedule dependency of combination treatment with L-asparaginase (A-Ase) and methotrexate (MTX) in leukemic mice; 8 treated mice per group and 20 untreated controls [*from* ref 104].

TABLE 28.—Effect of sequential treatment with adriamycin and methotrexate on the survival time of leukemic (L1210) mice* [*from* ref 103]

Optimal dose of adriamycin, Day 3 only (mg/kg)	Optimal dose of methotrexate, Day 4 to death (mg/kg)	MST (days)	%ILS
14	—	16	60
—	1.08	18.5	85
Specified dose(mg/kg)			
14	1.08	29	190
9	1.08	28.5	185
5	1.8	25.5	155
Untreated controls		10	

*BDF_1 mice inoculated ip with 10^5 L1210 ascites cells. Treatment ip.

synchrony of cell division. If synchronization with one drug can be followed by application of a second drug, such as an S-phase inhibitor, at a time when the tumor cells will be most susceptible, a therapeutic advantage may result. In one such attempt, partial cell synchronization occurred on treatment with demecolcine or vinblastine. Further treatment with cytosine arabinoside at the time when the cells were traversing S phase resulted in increased therapeutic response (107).

New drugs may be characterized as to their usefulness in remission induction or on loading-dose schedules as opposed to remission maintenance. With massive doses of a drug it is possible to reduce the tumor cell burden of advanced disease. This can be accomplished, for example, with a markedly cytotoxic drug, which can then be followed by more prolonged treatment with a second drug. Examples of loading-dose combination schedules are presented in table 31 (96).

TABLE 29.—Effect of combination chemotherapy on the emergence of resistance in leukemia L1210* [*from* ref 105]

Cell line	Test drug†		Transplantation generation 1	2	3	4	5
L1210/sensitive: no drug exposure	Cytosine arabinoside	OD‡	1400	1400	1400	1400	1400
		%ILS	122	118	100	111	128
	Cyclophosphamide	OD	200	300	200	300	300
		%ILS	128	> 200	117	> 200	> 200
L1210/combination: cytosine arabinoside (30 mg/kg, Days 1-5), cyclophosphamide (25 mg/kg, Days 1-5)	Cytosine arabinoside	OD	1400	1400	1400	2333	2333
		%ILS	150	106	117	111	77
	Cyclophosphamide	OD	100	300	300	300	100
		%ILS	106	> 200	> 200	> 200	27
L1210/cytosine arabinoside: cytosine arabinoside (30 mg/kg, Days 1-5)	Cytosine arabinoside	OD	1400	1400	833	2333	1400
		%ILS	138	111	75	5	11
L1210/cyclophosphamide: cyclophosphamide (25 mg/kg, Days 1-5)	Cyclophosphamide	OD	100	300	300	100	50
		%ILS	94	> 200	> 200	41	0

*10^5 L1210 cells implanted ip in groups of 8 BDF, male mice.

†Drug sensitivity measured to cytosine arabinoside (300-2333 mg/kg, q4d, Days 1,5,9) and cyclophosphamide (50-400 mg/kg, Day 1 only except for transplant generation 1 which was Days 1 and 5).

‡OD= optimal dose of test agent.

TABLE 30.—Therapeutic synergism against established Lewis lung tumor with a combination of cyclophosphamide plus methyl-CCNU [*from* ref 96]

Agent	Dose * (mg/kg)	ILS (%)	Day 75 S/T†
Cyclophosphamide	225	50	0/10
Methyl-CCNU	24	57	2/20
Cyclophosphamide + methyl-CCNU	150+ 24	> 168	16/20

*Single treatment Day 7. Local tumor 400 mg.

†S/T = No. of survivors/No. treated.

The effectiveness of new drugs can be examined with respect to their ability to stimulate G_0 cells to divide. This would make them highly useful in conjunction with a second drug that might more readily destroy the dividing cells.

The activity of new drugs may be explored in polychemotherapy. A model has been reported for combination chemotherapy with three drugs. This presented in table 32 and figure 33 (108). One study involving sequential administration of four drugs is shown in table 33 (14), where the activity of the four-drug combinations is compared with the drugs employed individually. Where polychemotherapy has demonstrated a therapeutic advantage over the

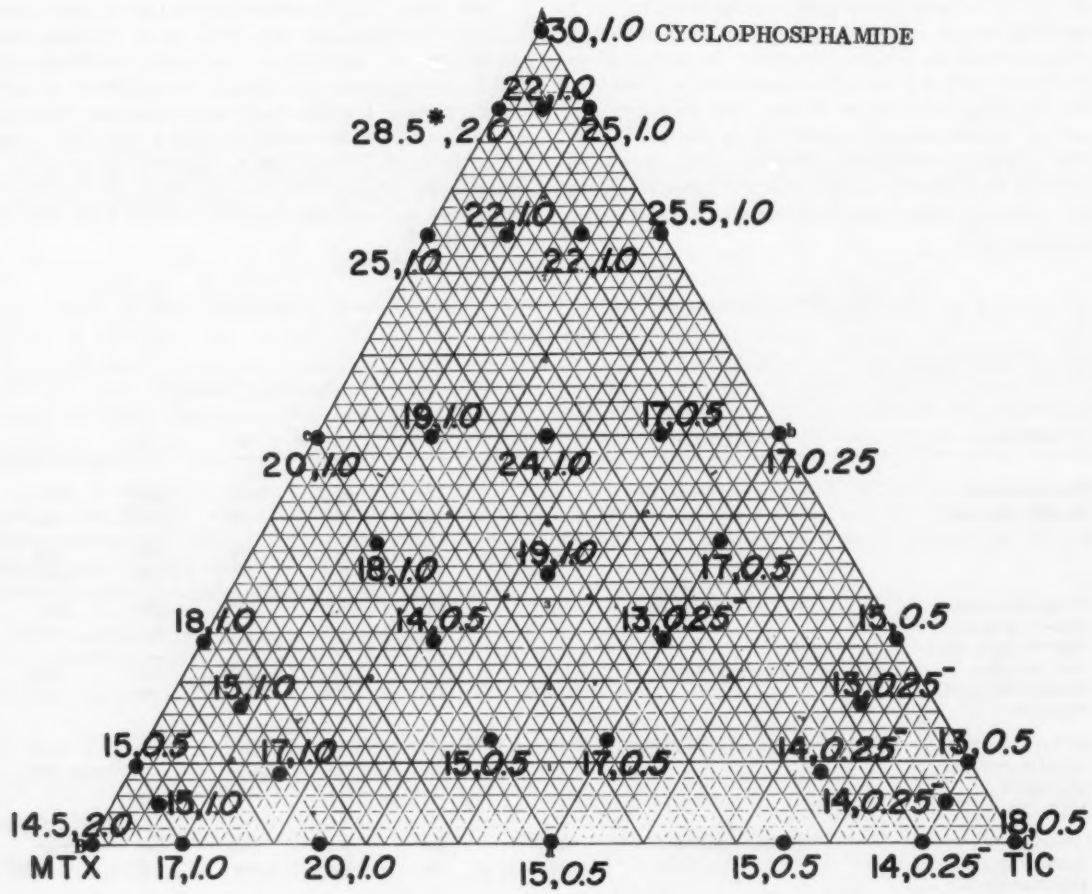


FIGURE 33.—Experimental design and results of testing a cyclophosphamide-methotrexate (MTX)-TIC combination. Each point on the chart identifies one of the relative proportions in which the 3 drugs were used. The numerical results shown adjacent to each point indicate the maximum MST attained with that relative proportion and the total dose (in terms of standard units given in the text of ref 108) yielding that MST. In a few instances, indicated by minus signs, the data were consistent with the possibility that the optimal dose could have been lower than any tested. An asterisk is used to indicate the occurrence of a long-term survivor or "cure" in the optimally treated group [*from* ref 108].

TABLE 31.—Therapeutic synergism on sequential treatment of advanced leukemia L1210 [*from* ref 96]

Priming drug	Second drug
6-MP	Methotrexate every 4 days
BCNU	6-MP every 2 days
Cyclophosphamide	Methotrexate daily or every 4 days
BCNU	Methotrexate daily, every 2 days, or every 4 days
5-FU	Methotrexate daily or every 4 days
Cyclophosphamide	Cytosine arabinoside 4 times daily
Cyclophosphamide	3′-Bromo-5′-chloromethotrexate every 4 days
Cyclophosphamide	3′,5′-Dichloromethotrexate every 4 days
BCNU	Cytosine arabinoside daily, every 2 days, or every 4 days

drugs employed individually, it is important to question whether any of the drugs used in the multiple chemotherapy are detracting from the net therapeutic response.

The potential applicability of experimental combination chemotherapy data to the clinic is illustrated in table 34 (109) where, for drugs demonstrated to be active individually against breast cancer, the available combination chemotherapy data are shown for the leukemia L1210 system. Such data may be helpful to the clinical investigator in choosing combinations for treatment of this disease in man.

J. Combined Modalities

A great deal of emphasis is being placed currently on the utilization of combined modalities for improvement of therapy. Preclinical investigation of new drugs in combined modalities including surgery, immunotherapy, and radiotherapy in conjunction with chemotherapy may provide information of importance for the clinic.

Surgery plus chemotherapy

An interesting model for studies in surgery plus chemotherapy is the one developed initially by Karrer et al (62,110,111) and utilized further at the Southern Research Institute (106,112). In this system, as described above, the Lewis lung tumor is inoculated im or sc in the hind leg and as the local tumor grows it metastasizes preferentially to the lungs. It is then possible to surgically remove the primary tumor mass (local tumor) by amputation of the hind leg and to then determine the influence of chemotherapy. In one such experiment (fig 25) (62) it was shown that surgery plus chemotherapy was more effective than surgery alone in increasing the lifespan of the animals. In another study (table 35) (106) it was observed that surgery plus treatment with cyclophosphamide plus methyl-CCNU was more effective than either surgery alone or the drugs individually or in combination in producing survivors of mice with advanced Lewis lung tumor. With other tumor systems, surgery plus chemotherapy has also been demonstrated to be more effective than either modality alone (113,114). In the utilization of surgery plus chemotherapy it is important to determine whether for specific drugs there is any advantage to pretreating with the drug relative to surgery, as opposed to concomitant treatment with surgery or treatment after surgery. On pretreatment it may be determined whether the drug is capable of causing sufficient regression to permit the extirpation of the remaining tumor by surgery. On posttreatment there is the possibility that the drug will be capable of total eradication of the reduced tumor cell mass, reaching areas where surgical procedures are ineffective.

Immunity plus chemotherapy

It is important to investigate the activity of new antitumor agents with respect to their use in immunochemotherapy and to clearly define the applicability to the clinic. A number of studies have appeared both at the preclinical and clinical level in which chemotherapy procedures are supplemented by treatment with nonspecific immunostimulants such as bacillus Calmette-Guérin (BCG) or *Corynebacterium parvum* (115,116). In animal studies involving the Moloney virus-induced leukemic subline LSTRA, it has been reported that chemotherapeutic treatment followed by administration of BCG resulted in greater increases in survival time and a greater number of survivors (fig 34) (117).

Adoptive transfer of immune lymphocytes has been employed in conjunction with chemotherapy in preclinical models and some success has been reported (118,119).

New drugs may be characterized with respect to their ability to alter the immunogenicity of tumor cells. Considerable evidence is accumulating showing that it is possible to alter the immunogenicity of tumor cells by treatment in vitro with substances such as neuraminidase (120) or in vivo by administration of antitumor agents such as DTIC (121). Such alteration of tumor cell immunogenicity may result in improved therapeutic response (collateral sensitivity) when accompanied by treatment with a chemotherapeutic agent (121,122,123). Evidence has been provided that on progressive treatment with DTIC over several transplant generations tumor cells become resistant. Not only do the tumor cells

TABLE 32.—Evaluation of combination treatment of leukemia L1210 with 3 drugs: A = cyclophosphamide, B = methotrexate, C = TIC* [*from* ref 108]

Dose (mg/kg) of drug—†			Fraction of anticipated individual optimal dose‡				
A	B	C	A (300 mg/kg)§	B (100 mg/kg)§	C (800 mg/kg)§	MST¶ (days)	Individual days of death**
600						11.5	8, 8, 8, 15, 27, 28
300			1.0	0	0	30	25, 28, 30, 31, 33
150						21	17, 20, 21, 22, 24
75.0						15	14, 15, 15, 15, 17
	200					14.5	11, 12, 14, 15, 17, 17
	100		0	1.0	0	14	12, 14, 14, 17, 17
	50.0					13	12, 13, 13, 13, 15
	25.0					14	12, 12, 14, 14, 14
		1600				8	8, 8, 8, 9, 9
		800	0	0	1.0	11	9, 11, 11, 11, 11
		400				18	11, 15, 18, 20, 21
		200				14	8, 14, 14, 15, 18,
540	20.0					28.5	8, 13, 27, 30, 31, >60
270	10.0		0.9	0.1	0	27	21, 25, 27, 28, 29
135	5.00					20	17, 20, 20, 22, >60
67.0	2.50					15	15, 15, 15, 17, 17
450	50.0					13	11, 13, 13, 13, 28, 29
225	25.0		0.75	0.25	0	25	22, 24, 25, 27, 29
112	12.0					20	15, 18, 20, 20, 21
56.0	6.00					15	15, 15, 15, 17, 17
300	100					13	12, 13, 13, 35, 51
150	50.0		0.5	0.5	0	20	20, 20, 20, 21, 22
75.0	25.0					17	17, 17, 17, 18, 19
37.0	12.0					14	13, 13, 14, 14, 15
150	150					17	12, 13, 17, 22, 32
75.0	75.0		0.25	0.75	0	18	17, 18, 18, 18, 20
37.0	37.0					14	13, 14, 14, 15, 17
19.0	19.0					13	12, 13, 13, 14, 15
60.0	180					14	12, 12, 14, 17, 18
30.0	90.0		0.1	0.9	0	14	12, 13, 14, 17, 17
15.0	45.0					15	13, 13, 15, 15, 17
7.50	22.0					13	12, 12, 13, 13, 17
540		160				8	8, 8, 8, 8, 8
270		80.0	0.9	0	0.1	25	22, 23, 25, 25, 31
135		40.0				18	17, 17, 18, 19, 20
67.0		20.0				15	14, 15, 15, 17, 17
450		400				8	8, 8, 8, 8, 8, 9
225		200	0.75	0	0.25	25.5	8, 9, 25, 26, 28, 39
112		100				20.5	18, 18, 20, 21, 29, 35
56.0		50.0				15	14, 14, 15, 15, 17
300		800				8	8, 8, 8, 8, 8
150		400	0.5	0	0.5	9	3, 9, 9, 9, 9
75.0		200				13.5	9, 9, 10, 17, 20, 21
37.0		100				17	14, 15, 17, 17, 17
150		1200				8	8, 8, 8, 8, 8
75.0		600	0.25	0	0.75	8	8, 8, 8, 8, 9
37.0		300				15	8, 11, 15, 15, 17
19.0		150				13	8, 13, 13, 14, 14
60.0		1440				8	8, 8, 8, 8, 8, 8
30.0		720	0.1	0	0.9	8	8, 8, 8, 8, 8
15.0		360				13	8, 9, 9, 17, 21, 21
7.50		180				13	8, 8, 13, 13, 17
	180	160				11	9, 10, 11, 11, 12
	90.0	80.0	0	0.9	0.1	17	15, 15, 17, 17, 17
	45.0	40.0				15	11, 14, 15, 15, 15
	22.0	20.0				13.5	12, 13, 13, 14, 14, 15
	150	400				8	8, 8, 8, 9, 17
	75.0	200	0	0.75	0.25	20	11, 15, 20, 20, 20
	37.0	100				13	13, 13, 13, 14, 15

TABLE 32.—Evaluation of combination treatment of leukemia L1210 with 3 drugs: A = cyclophosphamide, B = methotrexate, C = TIC* — Continued

Dose (mg/kg) of drug—†			Fraction of anticipated individual optimal dose‡				
A	B	C	A (300 mg/kg)§	B (100 mg/kg)§	C (800 mg/kg)§	MST¶ (days)	Individual days of death**
	19.0	50.0				13	13, 13, 13, 13, 13
	100	800				9	8, 9, 9, 9, 22
	50.0	400	0	0.5	0.5	9	8, 9, 9, 9, 9, 9
	25.0	200				15	8, 8, 15, 17, 17
	12.0	100				14	13, 13, 14, 15, 17
	50.0	1200				8	8, 8, 8, 8, 8, 8
	25.0	600	0	0.25	0.75	9	8, 9, 9, 17, 18
	12.5	300				15	9, 9, 15, 17, 17
	6.00	150				13.5	13, 13, 13, 14, 14, 15
	20.0	1440				8	8, 8, 8, 8, 8
	10.0	720	0	0.1	0.9	9	9, 9, 9, 9, 9, 11
	5.00	360				9	8, 9, 9, 9, 10
	2.50	180				14-	13, 13, 14, 17, 17
400	134	1056				8	8, 8, 8, 8, 8
200	67.0	528	0.33	0.33	0.33	8	8, 8, 8, 9, 9
100	33.0	264				19	8, 19, 19, 20, 21
50.0	17.0	132				15	14, 15, 15, 15, 17
30.0	180	80.0				13	11, 11, 12, 13, 13
15.0	90.0	40.0	0.05	0.9	0.05	15	14, 15, 15, 17, 17
7.50	45.0	20.0				14	13, 13, 14, 14, 14
3.70	22.0	10.0				13	13, 13, 13, 14, 14
540	10.0	80.0				8	8, 8, 8, 8, 15
270	5.00	40.0	0.9	0.05	0.05	22	21, 21, 22, 23, 24
135	2.50	20.0				18	17, 17, 18, 18, 18
67.0	1.20	10.0				15	15, 15, 15, 17, 17
30.0	10.0	1440				8	8, 8, 8, 8, 8
15.0	5.00	720	0.05	0.05	0.9	9	9, 9, 9, 9, 20
7.50	2.50	360				10	8, 9, 10, 12, 17
3.70	1.20	180				14-	13, 14, 14, 15, 18
50.0	150	268				12	11, 12, 12, 12, 13
25.0	75.0	134	0.083	0.75	0.167	17	17, 17, 17, 17, 18
12.0	37.0	67.0				15	13, 14, 15, 17, 17
6.00	19.0	33.0				12	12, 12, 12, 15, 17
100	150	132				13	12, 12, 13, 14, 25
50.0	75.0	66.0	0.167	0.75	0.083	15	15, 15, 15, 17, 17
25.0	37.0	33.0				15	14, 14, 15, 15, 17
12.0	19.0	16.0				13	12, 12, 13, 14, 15
450	17.0	268				9	8, 8, 9, 10, 30
225	8.50	134	0.75	0.083	0.167	22	17, 21, 22, 25, 26
112	4.20	67.0				17	15, 17, 17, 17, 19
56.0	2.10	33.0				13	12, 13, 13, 14, 14
450	33.0	132				8	8, 8, 8, 9, 13
225	17.0	66.0	0.75	0.167	0.083	22	20, 21, 22, 25, 25
112	8.50	33.0				17	17, 17, 17, 18, 18
56.0	4.20	16.0				13	13, 13, 13, 14, 15
50.0	33.0	1200				8	8, 8, 8, 8, 8
25.0	17.0	600	0.083	0.167	0.75	9	8, 8, 9, 9, 9
12.0	8.50	300				8	8, 8, 8, 8, 9
6.00	4.20	150				14-	12, 13, 14, 14, 18
100	17.0	1200				8	8, 8, 8, 8, 8
50.0	8.50	600	0.167	0.083	0.75	9	8, 8, 9, 9, 10
25.0	4.20	300				9	8, 8, 9, 9, 17
12.0	2.10	150				13-	8, 13, 13, 15, 15
75.0	100	600				8	8, 8, 8, 24, >60
37.0	50.0	300	0.125	0.5	0.375	9	8, 9, 9, 12, 18
19.0	25.0	150				15	9, 14, 15, 17, 17
9.00	12.0	75.0				13	13, 13, 13, 13, 14
150	100	400.0				8	8, 8, 8, 8, 10
75.0	50.0	200.0	0.25	0.5	0.25	10	8, 9, 10, 20, 24

TABLE 32.—Evaluation of combination treatment of leukemia L1210 with 3 drugs: A = cyclophosphamide, B = methotrexate, C = TIC*—Continued

Dose (mg/kg) of drug—†			Fraction of anticipated individual optimal dose‡				
			A (300 mg/kg)§	B (100 mg/kg)§	C (800 mg/kg)§		
A	B	C				MST¶ (days)	Individual days of death**
37.0	25.0	100				14	14, 14, 14, 14, 17
19.0	12.0	50.0				14	12, 13, 14, 14, 14
225	100	200				12	8, 8, 12, 12, 12
112	50.0	100	0.375	0.5	0.125	18	17, 17, 18, 19, 21
56.0	25.0	50.0				15	15, 15, 15, 18, 18
28.0	12.0	25.0				13	12, 13, 13, 14, 15
300	25.0	600				8	8, 8, 8, 24, 30
150	12.0	300	0.5	0.125	0.375	11	8, 8, 11, 15, 21
75.0	6.00	150				17	8, 17, 17, 17, 20
37.0	3.00	75.0				14	13, 13, 14, 15, 15
300	50.0	400				8	8, 8, 8, 9, 10
150	25.0	200	0.5	0.25	0.25	24	19, 20, 24, 24, 30
75.0	12.0	100				15	15, 15, 15, 17, 17
37.0	6.00	50.0				14	13, 13, 14, 15, 17
300	75.0	200				17	8, 8, 17, 29, 29
150	37.0	100	0.5	0.375	0.125	19	18, 19, 19, 20, 21
75.0	19.0	50.0				15	15, 15, 15, 17, 18
37.0	9.00	25.0				14	13, 14, 14, 15, 17
75.0	75.0	800				8	8, 8, 8, 8, 8
37.0	37.0	400	0.125	0.375	0.5	8	8, 8, 8, 8, 8
19.0	19.0	200				17	8, 9, 17, 17, 19
9.00	9.00	100				14	13, 13, 14, 14, 14
150	50.0	800				8	8, 8, 8, 8, 9
75.0	25.0	400	0.25	0.25	0.5	9	8, 8, 9, 9, 10
37.0	12.0	200				8	8, 8, 8, 9, 17
19.0	6.00	100				13-	12, 13, 13, 13, 14
225	25.0	800				8	8, 8, 8, 8, 9
112	12.0	400	0.375	0.125	0.5	9	8, 8, 9, 22, 26
56.0	6.00	200				17	8, 17, 17, 17, 19
28.0	3.00	100				14	13, 14, 14, 17, 20
Methotrexate	3.00					24	17, 24, 24, 26, 28
(standard):	1.50					28	24, 25, 28, 29, 33
daily treatment	0.75					31	26, 31, 31, 31, 33
	0.37					13	13, 13, 13, 14, 14
Untreated controls						11	10, 11 (13 deaths), 12 (2 deaths)

*Sc treatment given only on the 7th day after sc inoculation of leukemic spleen. Each mouse received 0.2 ml of saline containing 6×10^6 cells. BDF_1 male mice (19-25 g) were employed: 5 or 6 mice in treated groups; 16 mice in untreated groups.

†Vehicles employed for each drug were as follows: 0.85% saline solution for cyclophosphamide, 2% $NaHCO_3$ solution for methotrexate, and distilled water suspension for TIC.

‡ Division of dose into fractions is shown only in cases where the total is unity.

§ Anticipated individual optimal dose.

¶ Minus sign indicates that the optimal dose may have been lower than any employed.

** All 60-day survivors (> 60) were without palpable tumors.

TABLE 33.—Selection of anticancer agents: sequential treatment of advanced leukemia L1210 with 4 clinically active drugs, each drug administered sc for 3 days* [*from* ref 14]

Sequential treatment schedule with 4 drugs†				MST	Range of death	Relative increase in MST
Days 6-8	Days 9-11	Days 12-14	Days 15-17	(days)	(days)	(MTX = 100)
MTX	Me-GAG	CYC	6-MP	27	25-29	156
MTX	Me-GAG	6-MP	CYC	25.5	15-27	143
MTX	CYC	Me-GAG	6-MP	23.5	11-27	126
MTX	CYC	6-MP	Me-GAG	19	10-27	87
MTX	6-MP	Me-GAG	CYC	14	11-26	43
MTX	6-MP	CYC	Me-GAG	12	11-26	26
Me-GAG	CYC	6-MP	MTX	26.5	26-30	152
Me-GAG	6-MP	CYC	MTX	26.5	12-29	152
Me-GAG	CYC	MTX	6-MP	25.5	19-27	143
Me-GAG	6-MP	MTX	CYC	25	12-35	139
Me-GAG	MTX	CYC	6-MP	14	13-80	43
Me-GAG	MTX	6-MP	CYC	14	11-23	43
6-MP	CYC	MTX	Me-GAG	25	22-26	139
6-MP	CYC	Me-GAG	MTX	23.5	17-27	126
6-MP	MTX	Me-GAG	CYC	23.5	10-28	126
6-MP	MTX	CYC	Me-GAG	23	18-26	122
6-MP	Me-GAG	CYC	MTX	18	18-23	78
6-MP	Me-GAG	MTX	CYC	16	16-25	61
CYC	MTX	Me-GAG	6-MP	24.5	21-27	135
CYC	Me-GAG	6-MP	MTX	23	15-28	122
CYC	Me-GAG	MTX	6-MP	22.5	18-26	117
CYC	6-MP	Me-GAG	MTX	22.5	19-25	117
CYC	MTX	6-MP	Me-GAG	22	20-25	113
CYC	6-MP	MTX	Me-GAG	—	‡	
Daily to death, alone at optimal dose						
MTX (1.0 mg/kg)				20.5	17-26	100
CYC (25.0 mg/kg)				24.5	19-29	135
Me-GAG (50.0 mg/kg)				17	12-17	70
6-MP (100 mg/kg)				15	14-18	52
Untreated controls				9	9-10	

*Treatment schedule, Days 6-17. BDF_1 male mice (18-25 g) were employed; 10 mice per group, 20 untreated controls.
†Methotrexate (MTX) = 1.0 mg/kg; cyclophosphamide (CYC) = 50 mg/kg; methyl-GAG (Me-GAG) = 50 mg/kg; 6-MP = 100 mg/kg.
‡Not tested.

become resistant but also their antigenicity is altered so that eventually the tumor will not grow unless there is treatment with DTIC (121). Resistance to DTIC protects the tumor against the drug and the immunosuppressant activity of DTIC permits its growth. When the DTIC-resistant subline is subjected to treatment with BCNU marked collateral sensitivity is observed as expressed in increased sensitivity of the tumor to this drug (121). It has been demonstrated as indicated above that a variety of other sublines of leukemia L1210 resistant to various antitumor agents show collateral sensitivity to BCNU (58). Further investigation of the action of drugs on tumor cell antigenicity may lead to the choice of drug sequences of value for clinical application.

Radiation plus chemotherapy

New drugs can be tested in combination with radiation. There have not been extensive preclinical investigations with radiation plus chemotherapy and the full potential of this combined modality remains to be developed. In one study (fig 35) (124) it was shown that whole-body irradiation plus chemotherapy was more effective than either modality alone in increasing the survival time of mice with leukemia L1210. Wodinsky et al (125) have demonstrated that the administration of ten clinically active agents to mice with leukemia P388 in conjunction with γ-radiation was more effective than the drugs or γ-radiation given alone. Other studies may

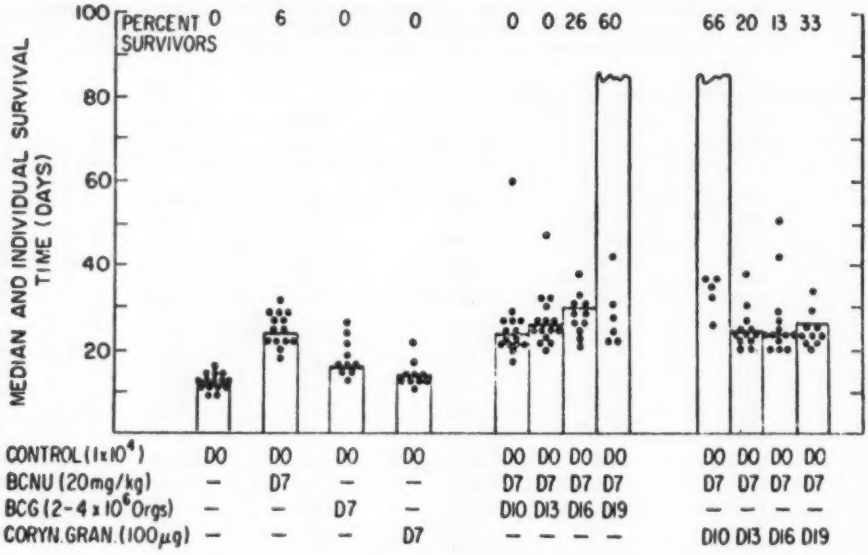

MEDIAN AND INDIVIDUAL SURVIVAL TIME (DAYS)
100
80
60
40
20
PERCENT SURVIVORS
0 6 0 0 0 0 26 60 66 20 13 33
CONTROL (1x10⁴) D0 D0 D0 D0 D0 D0 D0 D0 D0 D0 D0 D0
BCNU (20mg/kg) – D7 – – D7 D7 D7 D7 D7 D7 D7 D7
BCG (2-4 x 10⁶ Orgs) – – D7 – D10 D13 D16 D19 – – – –
CORYN. GRAN. (100μg) – – – D7 – – – – D10 D13 D16 D19

FIGURE 34.—Combined chemoimmunostimulant therapy of LSTRA. BCNU and stimulator given alone or in combination at specified time intervals following sc inoculation of 1×10^4 LSTRA cells on Day (D) 0. Each point represents time of death of individual animals (15 mice/group). Columns = MST for each group. Coryn Gran = *Corynebacterium granulosum* [*from* ref 117].

also be cited (126-128). Program development in this area should be forthcoming and additional preclinical models should be investigated that have particular pertinence for clinical application. As for combination chemotherapy and other combined modalities optimal schedules of administration should also be determined and the question of radiation given before chemotherapy as compared with radiation given concomitant with or after chemotherapy should be explored.

Obviously, for combined modalities it is possible to examine the total gamut of interrelationships of drug combinations, surgery, radiation, and immunotherapy with respect to the optimization of net therapeutic outcome. Detailed investigations should be conducted for the development of these most important areas.

K. Special Test Models

It is important to identify suitable model systems for characterization of drug activity against specific tumor categories in man. Models may be developed, for example, in which corresponding tumor types in animals are localized primarily in the same organs as in man. Systems such as Lewis lung carcinoma may be useful for drug identification and also for characterization of activity against lung tumor in man. Similarly, tumor systems such as the $CD8F_1$ (129) or C3H spontaneous mammary tumor in the mouse (130,131) or the 13762 mammary tumor system in the Fischer rat (132) may be useful for drug screening and evaluation for breast tumor in man. If appropriately developed, various types of human tumors growing in athymic mice (133) or thymectomized irradiated mice (134), including those of the lung, breast, gastrointestinal tract, brain, etc, could be applied to the study of drug activity in relation to clinical utilization. Development of chemotherapeutic and combined modality approaches with new drugs employing human and animal tumors in the immunologically deprived host is worthy of great emphasis. Drug activity in such new and novel systems may also be compared with the activity in the standard models employed in a tumor spectrum as well as with the activity in other systems.

The Ridgway osteogenic sarcoma provides a model which lends itself to clinical staging (135-137). The response to new drugs can be determined in this system with treatment initiated against early or advanced sc inoculated disease. Measurements may be made of the influence of the drug on parameters such as extent of tumor regression (complete and partial); duration of regression, median lifespan, and range of deaths in the animals that relapse; and increase in lifespan of the animals. The "cure" response can be determined. In this type of system it is possible to follow the influence of therapy on the individual mouse and its tumor. In one experiment illustrated in table 36 (ref 136; Schabel FM, Jr, unpublished data)

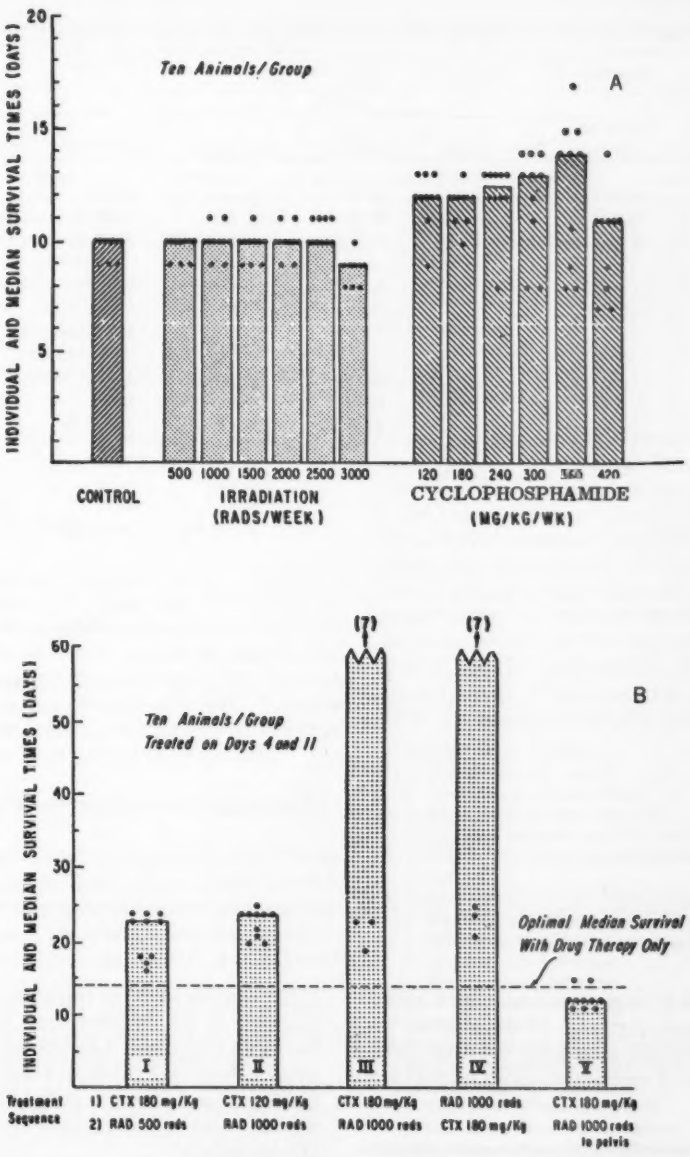


FIGURE 35.—*A.* Median and individual survival times for animals receiving no treatment, irradiation only, or cyclophosphamide (CTX) only. Circles = day of death of individual mice. *B.* Median and individual survival times for animals receiving combined systemic cyclophosphamide and central nervous system irradiation. RAD = radiation; circles = day of death of individual mice [*from* ref 124].

TABLE 34.—Two-drug combination chemotherapy studies in leukemia L1210 using drugs active in breast cancer [*from* ref 109]

	Maximum ILS at optimal dose of drug A, drug B, and combination of A and B*							
	Drug B							
Drug A	Adriamycin	Cyclophos-phamide	Methotrexate	5-Fluorouracil	Vincristine	Vinblastine	ThioTEPA	Melphalan
Adriamycin		A: 59 B: 200 A + B: > 300	A: 64 B: 45 A + B: > 300	A: 46 B: 72 A+B: 74	A: 68 B: 36 A + B: > 300	A: 43 B: 28 A+B: 63	A: 40 B: 60 A + B: 76	A: 104 B: 100 A+B: >300
Cyclophos-phamide	+		A: 166 B: 98 A+B: 234	A:> 300 B: 67 A + B: > 300	A: >300 B: 25 A+B: 100	A : > 300 B: 33 A + B: 150	A: 95 B: 27 A + B: 167	A: 108 B: 72 A+B: 175
Methotrexate	+	+		A: 111 B: 78 A+B: 25	A: 23 B: 11 A + B: 15	A: 110 B: 20 A + B: 72	A: 84 B: 54 A + B: 86	A: 102 B: 62 A+B: 112
5-Fluorouracil	–	–	+		A: 44 B: 28 A + B: 44	A: 72 B: 41 A + B: 68	A: 44 B: 28 A + B: 39	A: 72 B: 60 A+B: 82
Vincristine	+	–	–	–		A: 8 B: 17 A + B: 21	A: 33 B: 55 A + B: 55	A: 22 B: 78 A+B: 88
Vinblastine	±	–	–	–	–		A: 33 B: 59 A +B: 45	A: 33 B: 55 A+B: 61
ThioTEPA	±	+	–	–	–	–		A: 52 B: 61 A+B: 83
Melphalan	+	+	±	±	±	–	±	

*+ = Therapeutic synergism; – = no definite synergism.

it was observed that a single treatment with adriamycin (15 mg/kg) administered 12 days after implantation of the Ridgway osteogenic sarcoma yielded a high percentage of regressions (two partial and eight complete among ten animals) and an extensive increase in the survival time of the animals. When the same treatment was withheld until the 17th day after tumor implantation there were only four partial regressions and no complete regressions (among ten animals) or any increase in the survival time of the animals.

TABLE 35.—Response of Lewis lung carcinoma to surgery plus methyl-CCNU plus cyclophosphamide [*from* ref 96]

Treatment*	Percentage of long-term survivors	
	Chemotherapy alone	Chemotherapy + surgery
Methyl-CCNU	1	30
Cyclophosphamide	0	35
Methyl-CCNU + cyclophosphamide	24	70

*Treatment on the 12th day after sc implantation.

A number of experimental viral-induced tumors are now available for investigation of new drugs. Evidence has been obtained both in the Moloney leukemia system and in the spontaneous AKR leukemia system that eradication of the primary disease by chemotherapeutic agents can be accomplished, but that reinduction of the disease may occur as the result of the continued oncogenic action of the virus (138,139). This indicates the important need for investigations of the action of the new drugs with respect to their ability to suppress or prevent reinduc-

TABLE 36.—Treatment of established Ridgway osteogenic sarcoma (treatment ip Day 12 after implant) [*from* ref 136 and unpublished data of Schabel FM, Jr]

Drug	Dose (mg/kg)	No. of regressions/ total treated		Median duration of CR‡ (days)	No. of "cures"/ total treated
		Partial*	Complete†		
Actinomycin D	0.6	—	8/8	—	7/8
Adriamycin	14.0	—	7/8	11	0/8
Cyclophosphamide	156	—	8/8	21	3/8
Melphalan	10	1/8	7/8	28	4/8

* > 50% reduction in original tumor.
†Regression to below palpable size (< 65 mg).
‡In animals that relapsed after complete remission.

tion of disease either alone or in combination modalities and also their utilization in prophylaxis.

L. Carcinogenic Activity

In view of observations that many antitumor agents, when employed for an extended period of time, may exert carcinogenic action, it becomes important to examine all new antitumor agents for their mutagenic and carcinogenic activity. Various models are available for this purpose (140). It would also be of great importance to determine whether such carcinogenic activity could be controlled.

M. Chronobiologic Influences on Drug Activity

The area of chronobiology, of general importance in biology and medicine (141-143), is being investigated with respect to applicability to cancer therapy (144,145). Increased tolerance and improved response of mice with leukemia L1210 to treatment with cytosine arabinoside was reported when schedules were adjusted to the circadian system (146). Chronobiologic investigations of the action of new drugs may lead to improved drug effectiveness.

Specific attention could be focused on the application of the above and other methods to the treatment of the signal tumors in man, including breast, ovarian, lung, gastrointestinal, etc. Specific models should be developed that will provide information on new drugs that may be of value in the treatment of specific categories of neoplasia in man.

IN VITRO CHARACTERIZATION OF DRUGS

The in vitro methods employed for the screening and characterization of antitumor agents and their applicability for antitumor drug evaluation were originally reviewed in the comprehensive Gellhorn-Hirschberg report (147). In this analysis it was concluded that no in vitro system could replace an in vivo system for predictability of clinical antitumor activity. This conclusion undoubtedly still remains valid today, since in vitro systems in general examine a single response parameter, not taking into account the requirement of selective action against tumor within a host-tumor milieu. Nevertheless, the importance of in vitro characterization of new drug activity should not be underestimated, and the potential for the applicability of future developments remains real. Some areas of specific interest for in vitro development and potential application include:

a. Mammalian cell culture
   1. Growth inhibition assay
   2. Cytotoxicity (cell kill) assay
b. Antimicrobial and antiviral tests
c. Inhibition of oncogenic transformation
d. Inhibition of embryonic development, cellular differentiation, and growth
e. Specific inhibition of enzymes such as:
   1. DNA-dependent DNA polymerase
   2. RNA-dependent DNA polymerase
   3. Dihydrofolate reductase
   4. Ribonucleotide reductase
f. Cell culture determination of inhibition of DNA, RNA, and protein biosynthesis
g. Detailed determination of biochemical mechanism of action
h. Determination of cytokinetic characteristics
   1. Cell cycle phase specificity
   2. Sensitivity of noncycling cells
   3. Effects on cell cycle progression
i. Development of in vitro resistant cell lines
   1. Mechanism of resistance
   2. Rapidity of development of resistance in vitro
   3. Utilization of sublines resistant to diverse agents for evaluation of cross resistance

APPLICATION OF METHODOLOGY TO THE SELECTION OF ANALOGS

A difficult area for decision making involves the selection of new analogs of known antitumor agents for clinical trial. There are a great number and variety of analogs of alkylating agents, purine and py-

rimidine antagonists, folic acid antagonists, dimethanesulfonates, benzimidazoles, and other classes of drugs which have demonstrated activity in antitumor test models but which remain "shelved" pending any demonstration of a basis for further interest. There are a number of preclinical criteria that may be employed for the selection of new analogs. In general these involve the application of standard methodologies, as described above, to provide data as a guide for selection. Overall, in order to justify interest in a new analog of a known antitumor agent, the analog must be shown to possess quantitative or qualitative superiority over the parent compound in pharmacologic, therapeutic, or toxicologic properties. In this determination, specific attention can be focused on those characteristics which are limiting for the parent compound currently in clinical use.

Some preclinical criteria for selection of new analogs for clinical trial may be listed as follows:

a. Greater activity in appropriate test systems relative to parent drug in clinical use
b. Diminished limiting or undesirable acute, chronic, or delayed host toxicity such as myelosuppressive action, gastrointestinal toxicity, etc
c. Broader spectrum of effectiveness against experimental tumors including transplantable, carcinogen-induced, or spontaneous
d. More extensive delay in origin of resistance and/or activity against tumors with acquired resistance to the parent drug
e. Therapeutic synergism when employed in combination with parent drug (implies different pharmacologic or biochemical action)
f. Greater penetration to intracranial or other sequestered sites; specific organ localization
g. Greater effectiveness in preventing metastasis or in treating metastatic tumor
h. Less immunosuppressive action
i. Greater action against oncogenic virus, viral transformation, or viral-induced tumors
j. More desirable pharmacologic characteristics such as:
   1. Greater maintenance of blood and tissue levels; depot form with steady release
   2. More satisfactory dosage form, re improved solubility, stability, etc
   3. Active on oral administration when parent drug is orally inactive
k. Reduced carcinogenicity
l. Superiority over parent compound in combined modalities such as with radiation or surgery

CONCLUSION

Although not intended to be exhaustive, the current review illustrates the types of systems currently available for drug characterization following the initial demonstration of activity for a new compound in an antitumor screening program. New compounds could be characterized in a relatively rapid systematic manner in accordance with planned protocols to provide a broad base of data for decision making concerning further drug development and clinical investigation. These planned protocols could follow the same format and serve as an extension of the "Protocols for Screening Chemical Agents and Natural Products Against Animal Tumors and Other Biological Systems" (2).

LIST OF COMPOUNDS

N-acetyl-L-cysteine: NSC-111180; CAS reg. No. 616-91-1
Actinomycin D: NSC-3053
Adriamycin: NSC-123127; CAS reg. No. 23214-92-8
Aminopterin: NSC-739; CAS reg. No. 54-62-6; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl]methyl]amino)benzoyl]-
L-Asparaginase: NSC-109229; CAS reg. No. 9015-68-3
5-Azacytidine: NSC-102816; CAS reg. No. 320-67-2; *s*-triazin-2(1*H*)-one, 4-amino-1-β-D-ribofuranosyl-
8-Azaguanine: NSC-749; CAS reg. No. 529-99-7; 7*H*-v-triazolo[4,5-*d*]pyrimidin-7-one, 5-amino-1,6-dihydro-
Azaserine: NSC-742; CAS reg. No. 115-02-6; serine, diazoacetate (ester), L-
6-Azathymine: NSC-3426; CAS reg. No. 932-53-6; *as*-triazine-3,-5(2*H*,4*H*)-dione, 6-methyl-
BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-
BIC: NSC-82196; CAS reg. No. 5034-77-5; imidazole-4-carboxamide, 5-[3,3-bis(2-chloroethyl)-1-triazeno]-
3'-Bromo-5'-chloromethotrexate: NSC-98579; CAS reg. No. 6914-12-1; glutamic acid, *N*-[3-bromo-5-chloro-4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-
Busulfan: NSC-750; CAS reg. No. 55-98-1; 1,4-butanediol, dimethanesulfonate; Myleran
9-Butyl-6-MP: NSC-19488; CAS reg. No. 6165-01-1; purine-6-thiol, 9-butyl-
Camptothecin: NSC-100880; CAS reg. No. 25387-67-1
Carbazilquinone: NSC-134679; CAS reg. No. 24279-91-2; *p*-benzoquinone, 2,5-bis(1-aziridinyl)-3-(2-hydroxy-1-methoxyethyl)-6-methyl-, carbamate (ester)
CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-
6-Chloropurine: NSC-744; CAS reg. No. 87-42-3
Citrovorum factor: NSC-3590; CAS reg. No. 6035-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5, 6, 7, 8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate
Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride
Daunorubicin: NSC-82151; CAS reg. No. 23541-50-6
Demecolcine: NSC-3096; CAS reg. No. 477-30-5; colchicine, *N*-deacetyl-*N*-methyl-; Colcemid
3',5'-Dichloromethotrexate: NSC-29630; CAS reg. No. 528-74-5; glutamic acid, *N*-[3,5-dichloro-4-[[(2,4-diamino-6-pteridinyl)methyl]-methylamino]benzoyl]-
DTIC: NSC-45388; CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU
Ftorafur: NSC-148958; CAS reg. No. 17902-23-7; uracil, 5-fluoro-1-(tetrahydro-2-furyl)-
Guanazole: NSC-1895; CAS reg. No. 503-88-8; 1,2,4-triazolidine, 3,5-diimino-
5-Hydroxypicolinaldehyde thiosemicarbazone: NSC-107392; CAS reg. No. 19494-89-4
Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1
ICRF-159: NSC-129943; CAS reg. No. 21416-87-5; 2,6-piperazinedione, 4,4'-propylenedi-, (+)-

Isophosphamide: NSC-109724; CAS reg. No. 3778-73-2; 1,3,2-oxazaphosphorine, 3-(2-chloroethyl)-2-[(2-chloroethyl)amino]tetrahydro-, 2-oxide

6-Isopropylthiopurine: NSC-38306; purine, 2-amino-6-(isobutylthio)-

Isoquinaldehyde, thiosemicarbazone: NSC-92188; CAS reg. No. 2365-26-6

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate

6-Mercaptopurine riboside: NSC-4911; CAS reg. No. 574-25-4; 9*H*-purine-6-thiol, 9-β-D-ribofuranosyl-

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

Methyl-GAG: NSC-32946; CAS reg. No. 24968-67-0; guanidine, 1,1′-[(methylethanediylidene)dinitrilo]di-, dihydrochloride, monohydrate

6-Methylmercaptopurine riboside: NSC-40774; CAS reg. No. 7143-67-1; 9 *H*-purine, 6-(methylthio)9-β-D-ribofuranosyl-, dihydrate

6-(Methylthio)purine, trihydrate: NSC-20105; CAS reg. No. 7248-15-9

Mitomycin C: NSC-26980; CAS reg. No. 50-07-7

NSC-1402; CAS reg. No. 5334-31-6; pyrazole-4-carboxamide, 3-amino-

NSC-39073: CAS reg. No. 28069-00-3; 2,3-butanediol, *meso*-1,4-bis-(2-chloroethylamino)-, dihydrochloride

NSC-87974: CAS reg. No. 13908-93-5; urea, 3-cyclohexyl-1-(2-fluoroethyl)-1-nitroso-

NSC-88106: CAS reg. No. 13909-13-2; urea, 1-(2-chloroethyl)-3-(2-norbornyl)-1-nitroso-

NSC-114924: CAS reg. No. 21466-00-2; pyrazole-4-carboxamide, 3-(3,3-dimethyl-1-triazeno)-

NSC-407347: CAS reg. No. 3413-72-7; imidazole-4-carboxamide, 5-(3-methyl-1-triazeno)-

Picolinaldehyde, thiosemicarbazone: NSC-729; CAS reg. No. 3608-75-1; pyridine-2-CATS

Procarbazine: NSC-77213; CAS reg. No. 491-88-3; *p*-toluamide, *N*-isopropyl-α-(2-methylhydrazino)-, monohydrochloride

Thiamiprine: NSC-38887; CAS reg. No. 5581-52-2; purine, 2-amino-6-[(1-methyl-4-nitroimidazol-5-yl)thio]-

6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-

ThioTEPA: NSC-6396; CAS reg. No. 52-24-4; phosphine sulfide, tris(1-aziridinyl)-

TIC: NSC-60339; CAS reg. No. 70-09-7; terephthalanilide, 2-chloro-4′,4′′-di-2-imidazolin-2-yl-

cl-TIC: NSC-38280; CAS reg. No. 1820-92-4; terephthalanilide, 2-chloro-4′,4′′-di-2-imidazolin-2-yl-, dihydrochloride

Triethylenemelamine: NSC-9706; CAS reg. No. 51-18-3; *s*-triazine, 2,4,6-tris(1-aziridinyl)-

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Kinetics and Tumor Models[1]

Linda Simpson-Herren[2,3,4]

SUMMARY

The kinetic behavior of a tumor mass does not accurately reflect the kinetic behavior of the cell population either in untreated tumors or after cytotoxic therapy. Kinetic parameters for half-gram Ridgway osteogenic sarcoma, Lewis lung carcinoma, and B16 melanoma are compared and utilized in the evaluation of therapy end points. A cell kill equal to 3 $\log_{10}$ results in 70% mass regression of Ridgway osteogenic sarcoma, 30% mass regression of Lewis lung carcinoma, and no mass regression of B16 melanoma. Increase in lifespan correlates well with the delay in tumor growth in Ridgway osteogenic sarcoma and reasonably well in B16 melanoma, but poorly in Lewis lung carcinoma. The relative sensitivity of different tumors to a given cytotoxic agent appears to depend upon the end point chosen for evaluation.

[Cancer Chemother Rep Part 2, vol 5:83-88, 1975]

Tumor growth, stasis, or regression is the net result of many dynamic processes involving cell production, cell loss, cell death and lysis, reutilization, reabsorption, waste disposal, etc. Each of these processes influences the kinetic behavior of the tumor, the response to therapy, and the interpretation of end points (with the exception of long-term cures) used for evaluation of efficacy of therapy. It then becomes necessary to distinguish between the kinetic behavior of the tumor mass (which depends upon all of the factors mentioned above) and the kinetic behavior of the cell population (which is primarily cell production). An understanding of both types of kinetics is necessary to evaluate the effects of therapy.

Three transplanted murine tumor models, Ridgway osteogenic sarcoma (ROS), Lewis lung carcinoma (LL), and B16 melanoma (B16), exhibit wide variations in their sensitivity to a spectrum of antitumor agents. Responses range from partial or complete regressions and some cures with a number of agents in ROS tumors to limited obvious response with the most effective agents in B16. In this report, the therapy end points used to evaluate these responses will be examined in light of our present knowledge of the cell population kinetics and the tumor mass kinetics.

Biologic Systems

The ROS tumor line was derived from a spontaneous inguinal mass which appeared in an AKm mouse in 1948. The line is maintained by sc passage in AKR mice. Trocar fragments (about 100 mg) are implanted sc into $AKD2F_1$ mice for chemotherapeutic trials and for kinetic studies.

LL arose spontaneously in 1951 as a carcinoma of the lung in a C57BL/6 mouse and is maintained as an sc tumor by serial passage of small tumor fragments into mice of the same strain. Trocar fragments (about 25 mg) are implanted sc into BDF_1 mice for experimental studies.

The original tumor of the B16 line arose spontaneously in 1954 on the skin at the base of the ear in a C57BL/6 mouse and is propagated in that line. Sc implants of trocar fragments into BDF_1 mice are used for all experimental studies. Several sublines with various histories of ip passage, repeated frozen storage, etc, have been utilized over a period of time. The incidence of metastasis is different for each line and the data now available indicate that the kinetic properties of the lines may also be different. The metastatic capability of B16 may be modified during transplantation (1) and may correlate with biochemical properties (2).

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Tumor Growth

The growth of each of these tumors (3) may be described by a Gompertz equation, ie, exponential growth which is, in turn, exponentially retarded. Retardation is minimal in the ROS system (fig 1) and extensive in both LL and B16. The growth curve for B16 is a historical curve; data from all sublines were pooled.

Tumor mass doubling times (table 1) were calculated from tangents to the Gompertz-fitted curves at the indicated tumor masses and reflect tumor growth at that instant in time. The doubling time of ROS increased less than twofold between 50 mg and 10 g while the doubling time of LL increased 14-fold over the same range. The growth curve for B16 did not reach 10 g due to tumor necrosis or host death, and the time required for tumor mass to double at any tumor size examined was less than found in the other two systems.

Tumor Morphology

The growth curves represent the average behavior of tumor mass over the measurable range of growth but do not well represent the kinetic behavior of the cell population unless the tumor is composed of solidly packed cells. Few tumors even approximate a solid mass of cells and the cellularity of many tumors changes with increasing mass. In an excellent study of C3H tumors, McCredie et al (4) found in a long-transplanted line that the percent of tumor volume occupied by cancer cells decreased from about 70% in 10-mg tumors to about 30% in 1-g tumors. In contrast, the cancer cell volume of spontaneous tumors decreased from 80% to about 65% over the same range.

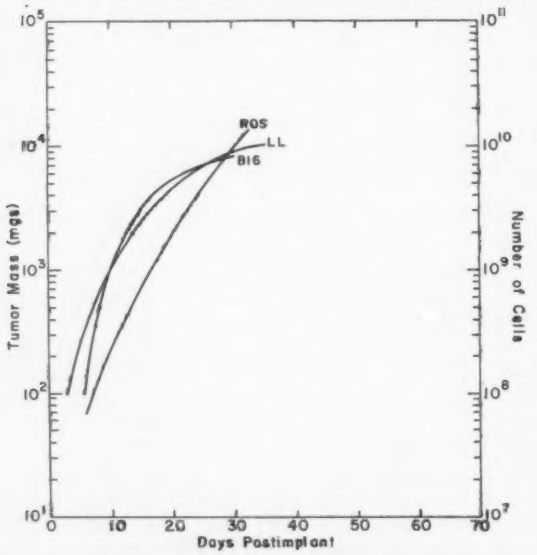

FIGURE 1.—Gompertz-fitted growth curves for ROS, LL, and B16.

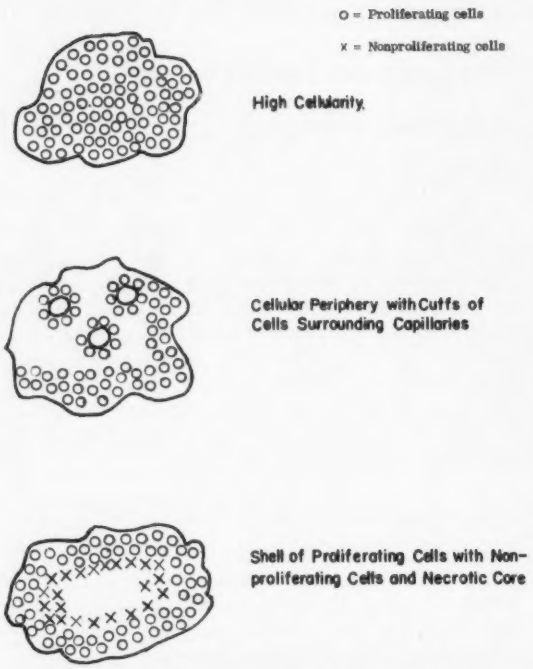
O = Proliferating cells
x = Nonproliferating cells
High Cellularity
Cellular Periphery with Cuffs of Cells Surrounding Capillaries
Shell of Proliferating Cells with Nonproliferating Cells and Necrotic Core

FIGURE 2.—Schematic representation of tumor morphology.

ROS, LL, and B16 tumors exhibit a variety of morphologic changes with increasing mass. Small ROS and LL tumors are highly cellular but are not solidly packed with cells. Small areas of necrosis are usually seen even at early stages of growth. With increasing mass, many ROS tumors show cording of viable cells around capillaries (*see* schematic diagram, fig 2). These cords or cuffs are surrounded by acellular material. Mitotic activity is usually seen throughout the cellular portion of the tumor.

Most LL tumors ulcerate at 1-1.5 g and become shells of viable cells surrounding nonproliferating

TABLE 1.—Tumor mass doubling times calculated from tangents to the Gompertz-fitted curves

Tumor weight (mg)	Doubling times (days) for—		
	ROS	LL	B16
50	2.5	1.5	0.9
100	2.6	1.7	1.0
500	3.1	2.5	1.6
1000	3.4	3.1	2.1
5000	4.2	7.7	7.6
10,000	4.8	21.1	—

cells (mitotic activity absent) and a necrotic core. Many B16 melanomas show massive necrosis at 0.5 g with only small areas of viable cells. These differences in cellularity may be attributed, at least in part, to vascularity of the tumor mass. In our experience it appears that ROS tumors are highly vascular while both B16 and LL rapidly outgrow their blood supply.

Effects of Tumor Morphology on Cell Population Kinetics

The changing tumor morphology affects the measurement of kinetic parameters. For instance, a thymidine index (TI) is based upon a representative sample of the "intact" cells and is not influenced by the acellular or frankly necrotic constituents of the tumor. Nonproliferating or dead cells which lyse rapidly have little effect on the measured index but those cells that persist intact for prolonged periods of time act as a diluent to the proliferating population and reduce the measured value of the TI. Thus, the rate of cell loss, either by lysis (and reutilization or reabsorption) or by physical removal of intact cells from the tumor, is an important factor in the observed kinetic parameters of the cell population.

By contrast, percent labeled mitoses (PLM) curves are based upon data obtained on cycling cells that enter mitoses during the period of observation and noncycling cells have little influence on the observed values. The situation now exists that of the two most important measurements currently being determined in cell population kinetics, one is essentially independent of the tumor mass behavior while the other may be dramatically affected by one or more of the dynamic processes which determine this behavior.

Cell Production

PLM curves were obtained on well-established (about 0.5 g mean weight) tumors for each of the three tumor systems and the data were analyzed by computer using the mathematic model described by Barrett (5) and Steel and Hanes (6). The curve for the ROS tumors (fig 3) has well-defined peaks and a very low minimum, both of which are consistent with small variations in the phases of the cell cycle and a narrow distribution of intermitotic times. The LL tumors (mean weight = 350 mg) have a shorter cell cycle with less well-defined peaks and a broader distribution of intermitotic times. The results of studies with B16 are far less clear. PLM data from an experiment performed in 1969 using our original low metastatic line are quite different from the data obtained recently on an sc line derived from a tumor carried by ip passage at Arthur D. Little, Inc, Boston, Mass. The latter line is highly metastatic, particularly to the lungs. The earlier PLM curve has a very flat second peak which is similar to many human tumor PLM curves (7). The more recent PLM curve has a higher and broader first peak as well as a better defined second peak and a narrower calculated distribution of intermitotic times. Other studies during the 6-year interval indicate that the original line and the isolated sublines of B16 have shown a gradual change toward a higher TI and a shorter cell cycle. The more rapid highly metastatic line we have described may have resulted from selection taking place during the adaption of the tumor to ip passage and then to sc passage. In contrast, the LL tumor and the sublines isolated from it over a period of 5 years have been relatively stable as far as the kinetics of cell population are concerned. Studies are in progress to attempt to clarify the observed differences in the B16 line.

Cell cycle parameters from the relatively small tumors of ROS, LL, and B16 are shown in table 2. A pulse exposure to tritiated thymidine (TdR-^{3}H) labeled 33% of the cells in ROS, 51% in LL, and 16%-41% (depending upon the experiment) in B16. The mean length of the cell cycle was 23.5 hours in ROS, 18.3 hours in LL, and 17.7 or 33.7 hours (depending

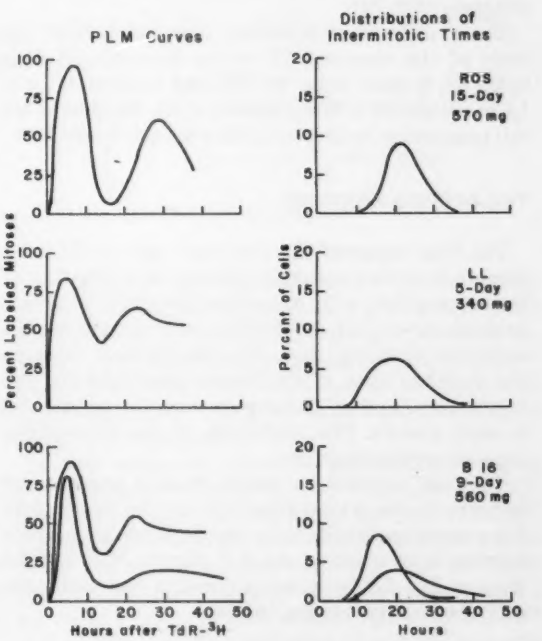

FIGURE 3.—PLM curves and distributions of intermitotic times obtained from computer analysis of PLM data.

TABLE 2.—Kinetic parameters of cell population and tumor mass

	Tumor line (days after implant)		
	ROS (15-day)	LL (5-day)	B16 (9-day)
Cell population parameters			
TI(%)	33	51	16-41
T_c (hrs)	23.5	18.3	17.7-33.7
T_s (hrs)	9.1	10.7	8.6-5.6
T_{G_1} (hrs)	12.0	5.2	6.9-25.5
Calculated proliferative fraction	1.0	0.9	0.9-1.0
Potential doubling time (days)	1.0	0.8	0.8-1.2
Tumor mass parameters			
Tumor weight (mg)	570	340	560
Doubling time (days)—			
From tangent	3.2	2.2	1.8
Experimental	3.3	2.5	2.1
Halving time (days)	2-4	3-4	—

upon the line) in B16. The G_1 phase occupied about 50% of the cell cycle in ROS and up to 75% in B16. Data from more advanced tumors indicate that both the TI and the length of the cell cycle remain essentially constant in ROS tumors up to 5 g or more[5] while the TI decreases and the length of the cell cycle increases with increasing mass in LL tumors (3). Studies of other tumor sizes and sublines are still in progress with B16.

The proliferative fraction, calculated from the ratio of the observed TI to the theoretic labeling index (8), is near unity in ROS and between 0.9 and 1.0 in LL and B16. The potential doubling time of the cell population is close to 1.0 day in each tumor line.

Tumor Mass Behavior

The time required for the total mass of ROS tumors to double exceeds the potential doubling time of the cell population by more than threefold. The mass doubling time of LL and B16 is two to three times the potential doubling time. The discrepancy between the doubling time of the tumor mass and the cell population appears to increase with increasing size in most tumors. The magnitude of the discrepancy depends on the tumor line.

The time required for tumor mass to regress also appears to be a characteristic of the tumor line. From experimental data, we have calculated a tumor halving time which is about 3 days in ROS and 3-4 days in LL. Little or no regression has been seen with single-dose therapy in B16.

[5]Simpson-Herren L. Unpublished data.

Relationships of Therapy End Points

Several therapy end points, including increased lifespan (ILS) and tumor mass regression, are used to evaluate the efficacy of noncurative therapy. In reviewing the results of chemotherapeutic studies from our laboratories, it soon became evident that mass regression of established tumors frequently follows therapy with several agents in ROS but is seldom, if ever, seen after the administration of single agents in B16. Wilcox et al (9) concluded that tumor mass behavior underestimated the cell kill. An ILS often occurred in mice with no evidence of tumor regression. We became concerned about the relationship of these end points and have attempted to utilize available kinetic information on tumors and cell populations as well as experimental therapy data to provide a basis for comparison.

Lloyd (10) has recently described a method of estimating the cell kill resulting from cytotoxic therapy if growth characteristics of the tumor are known. The delay in tumor growth, either to pretreatment size or to another arbitrary value, is a function of cell kill if the tumor growth characteristics are not modified by therapy. We have utilized this technique to estimate the cell kill in a number of experiments performed in our laboratory.

A schematic diagram of the effects of cell kill equal to 3 $\log_{10}$ in ROS, LL, and B16 is presented in figure 4. After a single dose of any agent that kills 3 $\log_{10}$ of cells, the ROS tumor mass will usually increase slightly and then regress about 70% (to about 30% of the pretreatment value). The median delay to time of recurrence to pretreatment size is 24.5 days. A similar cell kill in LL will result in only 30% regression and a delay of 13 days to recurrence.

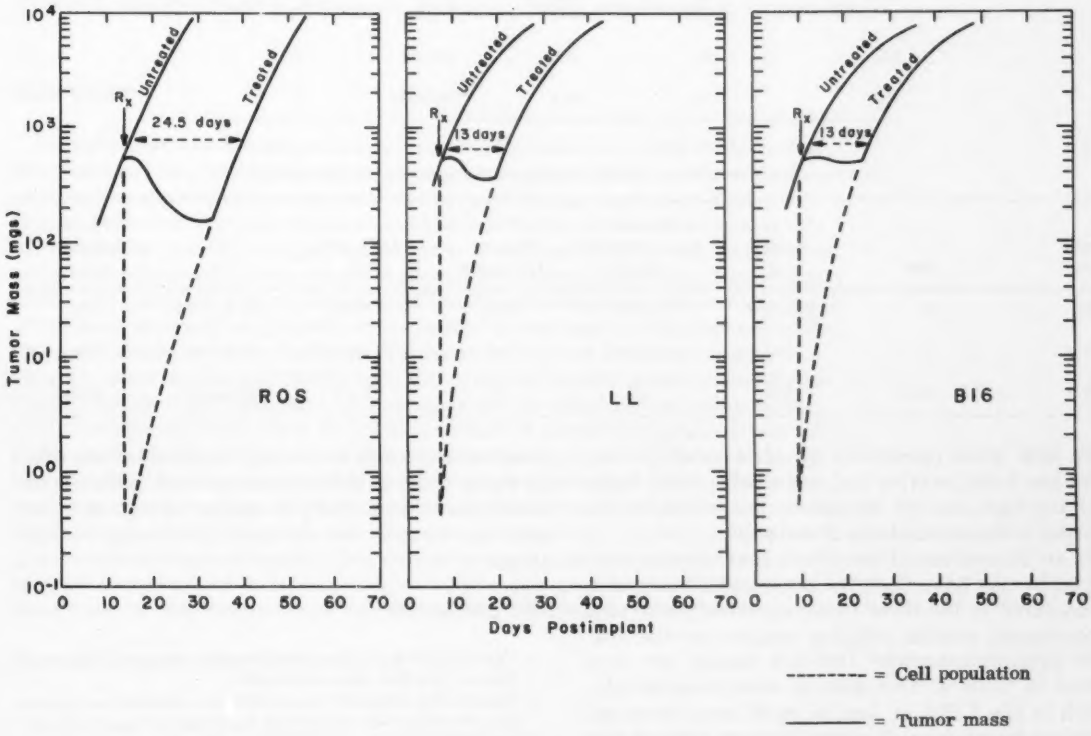

FIGURE 4.—Schematic representation of mass behavior following cell kill equal to 3 $logs_{10}$.

A cell kill of 3 $logs_{10}$ in B16 may result in no tumor regression and about 13 days' delay in tumor growth.

The long delay in tumor recurrence of ROS results from the relatively long doubling times of small tumors (0.5 g and less). This delay should provide a therapeutic advantage in multiple modality or combination therapy by allowing time for host recovery before additional therapy becomes necessary. Very small (0.5-500 mg) LL and B16 tumors appear to double much more rapidly than their more advanced counterparts and the reduction in cell number is quickly overcome.

The therapy end points shown in table 3 were obtained from experimental studies. The tumor or host response did not appear to be a function of either the cytotoxic agent or the tumor size in the range of 0.5-3 g; therefore, the data were pooled. This is not intended to imply that a given agent achieves the same cell kill at all stages of tumor growth or in all tumors; it only implies that the measurable tumor response to a given cell kill is a function of the tumor system, its kinetic characteristics, and, possibly, the tumor-host relationship.

A 50% mass regression of established (0.4 g or greater) tumors results from 1.5 $logs_{10}$ cell kill in ROS and 3-4 $logs_{10}$ cell kill in LL. ILS correlates well with cell kill in ROS but not in LL. Single-dose therapy of 0.4-g B16 tumors with the best available agents usually produces no mass regression but may kill up to 3 $logs_{10}$ of cells with a highly variable effect on lifespan. The estimates of cell kill are estimates of the cells killed in the primary tumor only. There is little evidence of invasion or metastasis

TABLE 3.—Relationship of therapy end points

Tumor line	Tumor regression (%)	Log_{10} cell kill	ILS (%)	Tumor growth delay (days)
ROS	50	1.5	50	14
	70	3.0	100	24.5
LL	50	3-4	20-100	13
B16	0	ca 3	Variable	13

TABLE 4.—Tumor response to a single dose of cyclophosphamide (200-225 mg/kg)

Tumor line	Day	Weight (mg)	Delay in tumor regrowth (days)	Percent regression	Log_{10} cell kill	ILS (%)	No. of cures/No. of mice
ROS	18	2000-3000	23-38	71-88	2.7-5.6	+90 to +122	0/40
LL	7	400	6-11	0-5	<1.0-2.0	−7 to +63	0/50
B16	10-11	350	5-8	0	0.7-1.5	+53 to +81	0/20

from ROS while essentially all established LL tumors are both invasive and metastatic. Data from both the high and low metastatic lines of B16 were included in the calculations of end points.

As an illustration of the effects that the choice of end point may have upon the measured efficacy of a given agent in the three tumor systems, data from experimental studies utilizing single-dose therapy with cyclophosphamide[6] (200-225 mg/kg) are presented in table 4. This dose of cyclophosphamide, which is the LD10 or less in each case, produces striking regressions of about 80% (to 20% of the pretreatment mass) in 2-3-g ROS tumors with 90%-120% ILS. The calculated cell kill in four experiments ranged from 2.7 to 5.6 $logs_{10}$. A similar dose of cyclophosphamide caused no regression of tumor mass but killed up to 2 $logs_{10}$ of cells in LL and up to 1.5 $logs_{10}$ in B16. Effects on lifespan in LL showed little correlation with the estimated cell kill in that tumor. The relative sensitivity of these systems to cyclophosphamide would depend upon the end point chosen.

CONCLUSIONS

Many processes, including the kinetic behavior of the cell population, contribute to the kinetic behavior of the tumor mass. Growth and regression rates of the tumor mass are characteristics of the tumor line, and the percent mass regression after therapy does not appear to be a useful measure of the relative sensitivity of different tumor lines to the agent. The apparent sensitivity of a tumor to a given agent may depend upon the end point chosen for evaluation.

[6]Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,-3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

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Solid Tumor Animal Model Therapeutically Predictive for Human Breast Cancer[1]

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SUMMARY

Data obtained with a spontaneous murine ($CD8F_1$) mammary tumor indicate that this model may be a predictive tool to investigate drug scheduling, immunotherapy, combination chemotherapy, and combined modality therapy for human breast cancer. The tumor-host system can be studied in different forms: (*a*) as an early or advanced slow-growing first generation tumor transplant in either sex; and (*b*) as an advanced spontaneous disease model, a spontaneous surgical adjuvant chemotherapy model, and a spontaneous metastases model in the autochthonous female host. Eleven of 12 agents shown to be clinically active against human cancer (list prepared by the National Cancer Institute, Bethesda, Md) are active against the animal tumor. Further, seven of eight agents clinically active against human breast cancer are active against the $CD8F_1$ mammary cancer. As in the clinical situation, surgical adjuvant chemotherapy with either the triple combination of cyclophosphamide plus adriamycin plus 5-fluorouracil or melphalan plus adriamycin plus 5-fluorouracil is more effective than any lesser combination or single agent alone. Immunologic parameters concerning host and the autochthonous tumor can be evaluated and can be critical in scheduling agents for cure. Immunochemotherapy (employing a nonspecific immunostimulant and combination chemotherapy against the autochthonous tumor) enhances cure rates. It seems likely that this slow-growing solid tumor model should also reveal information of value for human solid tumors other than breast.

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The data to be presented concerning the spontaneous breast carcinoma arising in the $CD8F_1$ female mouse demonstrate that this system represents a preclinical therapeutically predictive solid tumor animal model which can be used to investigate anticancer agents, drug scheduling, antimetastases therapy, combination chemotherapy, immunotherapy, and combined modality therapy for human breast cancer.

Although transplantable tumor systems have facilitated progress in cancer therapy, the search continues for animal models that will predict more accurately the activity of anticancer drugs in man.[5] Transplantable tumors differ in important respects from spontaneously arising neoplasms. Spontaneous animal tumors in autochthonous hosts have greater qualitative heterogeneity in their neoplastic cell populations (99,132,130) than transplantable tumors which have both a more homogeneous tumor cell population and a faster growth rate than primary tumors. In addition, whereas transplantable tumors have a high growth fraction (120, 129), primary spontaneous cancers (human or animal) grow slowly, in large part due to a low growth fraction.

Transplants, including those of spontaneous mammary tumors, can be expediently studied in

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[5]DeVita VT, Jr, and Rauscher FJ. Special communication; National Cancer Program, May 23, 1975.

syngeneic systems where multiple factors (eg, combination chemotherapy and/or immunotherapy) can be evaluated simultaneously with all experimentally proper groups and controls in large groups of animals. However, the ultimate questions are whether and how such factors interrelate in the spontaneous, autochthonous human tumor situation where human hosts make some type of subtle, complicated, slowly evolving, and long-standing immune response against their own cancers. For this reason, and because the more common human cancers are the *solid* types, which are the least responsive to treatment (58), the logical choice for a potentially predictive preclinical test model is a spontaneous solid cancer in its autochthonous animal host.

Most murine mammary carcinomas are spontaneous solid tumors that: (*a*) grow relatively slowly, as do human cancers; (*b*) have a viral etiology as is likely for human breast cancer (149); (*c*) metastasize (8) as do human cancers; (*d*) are relatively refractory to chemotherapeutic agents as are human cancers (186, 187, 63, 47, 90); (*e*) are immunogenic in their autochthonous hosts as are human cancers (107); and (*f*) can be used to evaluate combined modality therapy in their autochthonous hosts (134, 143, 144, 84) and can be employed for expedient testing as first generation transplants in large numbers of syngeneic hosts of reasonable cost. Therefore, one of the many murine mammary tumor systems is the obvious choice for evaluation as a predictive model.

Other artificial screening systems do not possess all the aforementioned characteristics. For example, some human tumors can be grown in cell culture, but without the adjacent normal cell-malignant cell and immune cell-malignant cell interactions that are present in the in vivo autochthonous situation. Hence, there are good reasons for doubting whether the response of a patient's tumor can be predicted by assaying the effects of drugs on cell cultures of that tumor. In vivo and in vitro conditions differ profoundly in ways that greatly influence drug effectiveness, and a lack of correlation between chemotherapeutic results in vivo and in vitro (63,10) has been shown with murine spontaneous mammary carcinomas. Human tumors also can be grown in heavily immunosuppressed mice (eg, thymectomized, irradiated, antilymphocyte serum-treated mice) or implanted into immunodeficient nude mice as xenogeneic hosts for evaluation of their response to chemotherapy. However, since complementary host immunity has been shown important for optimal chemotherapeutic effect (138, 133, 139, 40, 154, 144, 219, 84, 135),[6] such profound immunosuppressive conditions may well influence drug effect. Consequently, the results attained in these xenogeneic systems, rather than under true spontaneous autochthonous tumor conditions, would probably give erroneous predictions of the drug activity which can be achieved in man.

Additional caution is required in the interpretation of such xenogeneic data because certain histologic criteria (eg, percent mitoses) were found to change during growth in xenogeneic hosts. Also, the "takes" and growth rates of human tumor transplants are unpredictable and highly variable; eg, no human breast carcinoma grew well, some did not survive, and the remainder grew poorly for only 3-7 weeks (18). Xenogeneic model systems drastically change the host-cell interactions that are present under spontaneous, autochthonous conditions. Consequently, these new screening systems currently await fuller evaluation and validation as barometers of drug activity against human cancers. At present, the uncertain tumor transplant "takes" and their greatly varying growth rates under highly artificial conditions dictate caution in postulating that drug-positive correlations with clinical results will occur more often than by chance alone.

The mouse mammary tumor virus (MMTV) system is of special interest as a clinical test model because, as noted earlier, it shares the general characteristics of spontaneous neoplasms, including those in man. More specifically, numerous investigators have reported many similarities between murine and human breast cancer (158, 117, 206, 51, 111, 221, 152, 149, 150, 113, 218). These correlations provide important background knowledge in any consideration of the MMTV system as a predictive model and are therefore presented in the next several sections of this paper.

ANATOMIC-BIOLOGIC CORRELATIONS BETWEEN SPONTANEOUS MURINE AND HUMAN BREAST CANCER

Table 1 lists some general anatomic-biologic correlations. As noted, there are important similarities in morphologic characteristics, genetic factors, metastasizing relationships, and host resistance features. A more detailed presentation of correlations at the viral, hormonal, immunologic, and chemotherapeutic levels will be discussed in separate sections with accompanying tables.

Evidence for a Common Viral Etiology Between Spontaneous Murine and Human Breast Cancer

Viruses, both RNA and DNA, are now known to be involved in the origin of neoplasia of many types in

[6]Gutterman J. Unpublished observations, M.D. Anderson Hospital, Houston, Tex.

TABLE 1.—Some general anatomic-biologic correlations between spontaneous MMTV and human breast cancer

Key element	Correlation	Ref No.	
		Mouse	Man
Morphology	Multifocal diffuse disease (ie, second primary)	20,81,56,125	231,86-89,80,247
Genetics	Familial tendency	110,189,34,56,243, 66	95,37,229,7
Metastases	a. Present in both species	45,189,8	159,34,121,79
	b. Greater tumor size correlates with greater incidence of metastases		
Host resistance	a. Spontaneous regression	232,108,236,237, 155,156,246,29, 173,59,179, 218,251,5, 217,245	25-28,6,71,72,213, 9,100,121
	b. Evidence for tumor-specific antigens		

animals of many species. This biologic fact mandates that the concept of the viral etiology of cancer be considered a principal approach to the conquest of cancer (94).

In regard to the relationship of viruses to breast cancer per se, mammary neoplasms in mice have been proven to be due, in part, to the presence of a factor in milk (24), later identified as type B virus particles (127). As noted in table 2, electron microscopy has demonstrated these particles in MMTV as well as in the milk of such tumor-bearing animals (65). These agents differ in antigenicity, virulence, mode of transmission (eg, milk and/or sex cells), pathology of induced tumors, and host range (257, 151, 183, 160, 253, 243). The genotypic variations among mouse strains with respect to the spontaneous incidence and transmission of the virus, and the fact that the production of virus occurs only in lactating mammary glands and mammary carcinomas, contrasts sharply with the universal distribution of the viral structural genomes (17, 23). These strain and tissue specificities (ie, breast cancer) indicate that there are control mechanisms determined by both genetic (66) and hormonal (23) factors.

Thus, in addition to the presence of the MMTV itself, genetic constitution, hormonal stimulation, immune response, diet, nursing, and co-carcinogens have also been shown to be involved in the genesis of the tumor (164, 183). Mammary tumorigenesis in the mouse results from the interaction of several factors which may act either simultaneously or consecutively, and may vary quantitatively in their importance with the absence of some being substituted for by others of increased potency. For example, the MMTV can potentiate the endogenous prolactin effect on mammary gland differentiation, and this response varies among mouse strains due to genetic factors (66,16). Further, the mammary tumor can be induced by continuous hormonal stimulation of so-called low mammary-cancer mouse strains in the absence of detectable virus (109,35) as well as by irradiation or carcinogenic chemicals (91).

Similarly (table 2), human breast cancer demonstrates evidence which strongly supports the existence of a human breast cancer virus (149). As in mice, human breast cancer has also been demonstrated to be inducible by radiation (128), and the consistently demonstrated familial aggregation (229) provides some additional indirect support for a virus etiology of human breast cancer. The viral background takes on even greater significance in that virus particles, aside from having been demonstrated in human cancer and in human milk (table 2), have also been found on electron microscopy in mammary tumors of rats (43,32) and in a spontaneous mammary tumor of a rhesus monkey (44,2). Since the MMTV is at this time the only generally available viral agent of proven capacity to induce breast cancer, the murine system has been adopted as an experimental model for human breast cancer by viral biochemists (183).

The morphologic, biochemical, molecular biologic, and immunologic findings summarized in table 2 do not provide proof of viral etiology for human breast cancer. However, they do provide exciting evidence of virus-related information in human breast cancer revealing remarkable similarity and correlation with the corresponding virus-induced mammary neoplasia in mice and (en masse) compellingly implicate a virus as the causative agent of human breast cancer. However, it is necessary to show that the particles in human milk and human breast cancer

TABLE 2.—Common viral etiology of MMTV and human breast cancer (RNA tumor virus)*

Test	Correlation	Ref No. Mouse	Ref No. Man
Electron microscope			
Milk	Type B viral particles	24,127,65, 64	75,153,65,152, 76
Breast cancer	Type B viral particles		
Reverse transcriptase (unique to RNA tumor viruses)			
Milk	Present in both species	184,147	184,177
Breast cancer	Present in both species		
70S RNA (unique to RNA tumor viruses)			
Milk	Present in both species	147	147
Breast cancer	Present in both species		
Simultaneous detection test (for reverse transcriptase and 70S RNA)			
Milk	Present in both species	185,216	184,216
Breast cancer	Present in both species		
Molecular hybridization tests: homology between MMTV RNA and RNA of human breast cancer	1. DNA synthesized by human milk particles hybridizes with the RNA of human breast cancer	50,184	50,184
	2. MMTV-tritiated DNA *specifically* hybridizes with polysomal RNA from human breast cancer		
Immunologic correlates	A. Sera of women with breast cancer—		
	1. Neutralize MMTV	42	42
	2. Produce positive indirect immunofluorescence test with the cells of MMTV tumors	174	174
	3. Have anti-MMTV antibodies as shown on electron microscopy by peroxidose labeling of MMTV	114	114
	4. Have antigens that precipitate in gels with anti-MMTV antibodies	162	162
	B. Leukocyte migration tests reveal that human cancer has an antigenic similarity to MMTV	28	28

*Indirect evidence; direct evidence requires the demonstration that the particles found in human breast cancer produce the disease in a suitable experimental animal.

can produce the disease in a suitable experimental animal. The small amounts of particles thus far isolated from human breast cancer material have not yet made such final direct correlation feasible as a practical expectation. Nevertheless, the morphologic, immunologic, and biochemical correlations of viral particles in mouse and man are striking, particularly in relation to all the other parallels that can be drawn between murine and human breast cancer.

Relation of Hormones to Spontaneous Virus-Induced Murine Breast Cancer and Correlations With Human Breast Cancer

Carcinoma of the breast is a tumor of hormonally responsive tissue (table 3). The endocrine control of breast growth and function is extremely complex, involving many hormones including insulin, glucocorticoids, pituitary hormones, thyroxine, and estrogen. The most important appear to be estrogen and prolactin, both of which (but particularly prolactin) have been implicated as being involved in the development of breast cancer in mice and in man.

Although carcinoma of the breast arises in hormonally responsive tissue, it does not retain this hormonal sensitivity (table 3). More than half of all human breast cancers are initially hormone-independent tumors, and the remaining number of hormone-dependent tumors increasingly develop secondary hormonal independence during their course. Thus, some patients (30%-40%) with primary carcinoma of the breast have a dramatic, but temporary, remission when treated by endocrine ablative surgery or by steroid therapy (69). No completely satisfactory explanation has been accepted to account

TABLE 3.—Relationship of hormones to spontaneous virus-induced MMTV and correlations with human breast cancer

Key element	Correlation	Ref No.	
		Mouse	Man
Develops in hormonally responsive tissue	Prolactin, estrogen, growth hormone, insulin, glucocorticoids, growth and function in both species	165	227
Key hormone affecting tumor development	Prolactin in both species	159,189,97,21,163, 254,255,161	214,250,67,119
Hypothalamic lesions affect prolactin and effect breast tumors	Present in both species	36	214
Hormone-dependent, preneoplastic epithelial hyperplasia	Present in both species	165,254	214,247
Hormonal tumor characteristic	In mice majority are hormone independent; hormone-dependent tumors progress to hormone independence	211,146,217, 241, 3	
	In man 60% are hormone independent; hormone-dependent tumors progress to hormone independence		96,69
Correlation between estrogen receptor content and hormone-responsive growth	Present in both species	209	69,175
Effect of estrogen	Increases prolactin in both species	165	214

for the remission which sometimes results from the administration of estrogens, antiestrogens, androgens, or glucocorticoids.

Mice also develop both hormone-dependent and hormone-independent tumors, the percentage of which varies with the strain. All $CD8F_1$ females develop hormone-independent tumors. GR mice have hormone-dependent tumors in 100% of the breeding females (3) which regress after each pregnancy. However, the regression is less rapid with each succeeding pregnancy until eventually they grow in nonpregnant mice and are hormone independent. Sometimes, dependent tumors show a very rapid progression to pregnancy-independent or hormone-independent growth. Hormone-independent tumors may show residual responsiveness to pregnancy in that they generally grow more rapidly during pregnancy and may show partial regression post-partum (211). Of the tumors developing in strain DDD mice, roughly one third were hormone dependent (146). Hormone-dependent tumors are either rare or completely absent in either virgins or breeders that are older than 12 months in some mouse strains (217, 241). Thus, mice (as well as man) develop both hormone-dependent and hormone-independent mammary tumors, the majority of which are hormone-independent in most strains. In addition, hormone-dependent mammary tumors in mice progress to become hormone-independent, as do breast cancers in man (69).

Some murine and human breast cancer cells possess estradiol receptors, and an excellent correlation can be found between the ability of such tumor extracts to bind estradiol in vitro and the responsiveness of the tumor in vivo to endocrine ablation or steroid therapy (table 3). A recent study (175) reveals that 55% of women with breast cancer who had endocrine ablation therapy and estradiol positive (ER+) tumors showed objective remission as compared to only 8% who had estradiol negative (ER−) tumors. Similarly, of women with breast cancer who received steroid therapy, 60% of those with ER+ tumors showed tumor regression compared to only 8% of those with ER− tumors. Thus, human tumors containing estradiol receptors tend to respond to a change in hormonal milieu more than those without such binding sites, and likewise, mammary tumors in mice show a correlation between estrogen-receptor content and hormone-responsive growth (209).

Similarity of Immune Response in Human and Murine Breast Cancer

As summarized in table 4, cellular and humoral immune responses are similar in murine and human breast cancer. These responses appear to be significant since correlations between immunologic parameters with tumor growth, metastases, and regression have been found in both mouse and man. Therefore,

TABLE 4.—Similarity of immune response to human and murine breast cancer

Immunologic reactivity	Correlation	Ref No.	
		Mouse	Man
Cell mediated			
1. Cell-mediated immunity (T-cell reactivity)	Present in both species (tumor-specific and cross-reactive)	108,219,122, 105,232	107,101,100,178, 11,103,104
2. Antibody-dependent cellular immunity (B-cell reactivity)	Present in both species	222,171	103,104
Humoral mediated			
1. Complement-dependent cytotoxic antibody	Present in both species	220,116,223	54,11,100
2. "Unblocking" antibody	Present in both species	223	102
3. Blocking factor	Present in both species	105,223,106, 30,219,107 220	107,104

the therapeutic potential of immunotherapy appears very important. Chemotherapy, particularly combination chemotherapy, while often impressive in achieving regression of neoplastic disease (including prolonged remission) has accomplished cure only infrequently. Cure is difficult to achieve because of the failure to eliminate the last few residual viable tumor cells. There is good scientific rationale (140) to expect that the addition of effective immunotherapy to chemotherapy can selectively kill the last few cancer cells and result in cure. There are also firm supportive data for this expectation (132-135, 138, 139, 141, 143, 144, 145, 84, 40, 49, 72-74, 154, 157, 166-168, 172, 215,219, 240, 242).

It seems clear, therefore, that an understanding of the complexities of the immune response to an autochthonous tumor is important since the chemotherapeutic cure of cancer is dependent on the synergistic interplay of host defenses. Since the cure of cancer is the goal, it is essential to evaluate chemotherapy in a model system which lends itself to the normal operational interaction of immunity with chemotherapy—namely a spontaneous solid tumor in its autochthonous host.

$CD8F_1$ Model Tumor System

The animals employed are the first generation hybrid ($CD8F_1$) resulting from the cross-mating of inbred BALB/c females with inbred DBA/8 males. The hybrid females, infected maternally with the MMTV, have a high mammary tumor incidence (about 80%) arising in animals which are 9-10 months of age. The $CD8F_1$ hybrid strain was selected as a model system because the animals are healthier, hardier, and not prone to diarrhea as are other spontaneous mammary tumor-carrying strains. Consequently, these hybrids are better able to withstand anesthesia, surgery, adjuvant combination chemotherapy, and immunotherapy than are the inbred mice strains. Details of this animal colony have been published (221).

Methods of Evaluating Therapy on $CD8F_1$ Murine Mammary Tumors

As listed in table 5, there are five available testing methods; the first two employ a first generation tumor transplant, whereas the last three are reserved for definitive testing in the spontaneous tumor-bearing host. The choice of method depends upon the therapeutic testing problem.

Early First Generation Transplants

For preliminary screening of drug activity, the fastest and simplest method is obviously desirable. First generation transplants in syngeneic hosts are not far removed biologically from the parent spontaneous tumor and they are slow-growing tumors, taking about a month to reach an average size of 1 g. An "early" first generation transplant method initiates therapy on 24-hour transplants which, being very small tumors, are the most responsive to therapy; ie, the fewer the number of solid tumor cells, the greater the chemotherapeutic effect (134, 197,139,115). Therefore, this "early" transplant system is the most sensitive of the five methods in picking up anticancer activity. Concern that screening spontaneous murine mammary cancers will be unduly sensitive in yielding false positives is not warranted; aside from our own considerable experience, other extensive data on C3H mammary tumor systems have been reported attesting to their general resistance to chemotherapy (187,188,181). In the transplant systems, a single spontaneous mammary tumor is utilized for transplantation of each experiment to avoid variation that might be induced by the differing tumor-specific antigens of different spontaneous tumors. Since the hormone-independent tumor transplants grow equally well in both sexes, either male or female hosts may be employed. However, 2-4-month-old males are usually used in order to spare the females for spontaneous tumor development at 9-10 months of age.

This slow-growing tumor system lends itself to the evaluation of various schedules of drug administration. In general, our experience recommends a once-weekly therapy schedule to begin the day after transplantation in the "early" system. Drug effectiveness is determined by comparing wet tumor weights of control and treated tumors (T/C). The duration of time for initial evaluation of a potential agent in the "early" tumor system is approximately 1 month.

Advanced First Generation Transplants

Advanced tumor transplants (ie, first generation transplants in either males or females from a single spontaneous mammary tumor) are employed to evaluate combination chemotherapy begun after about 1 month of untreated tumor growth (average tumor size at institution of therapy is approximately 200 mg). If median survival time is the parameter used to determine drug effectiveness, the duration of advanced tumor experiments is about 2.5 months from transplantation, the time at which all controls have died tumor deaths. Shorter duration (eg, 2 months) for these experiments may be achieved by utilizing the parameter of inhibition or regression of tumor growth in treated animals as compared to that in untreated controls (tumor volume, T/C) to determine drug effectiveness.

TABLE 5.—Methods of evaluating therapy in $CD8F_1$ murine mammary cancer

Methods	Sex	"Takes" (%)	Average initial tumor wt at 1st treatment	Day injection began	Average No. of weekly injections*	Average tumor wt at time of sacrifice†	Median day of death by tumor†	Median day regrowth appears†	Duration of experiment (mos)
Early 1st generation transplants for initial screening	Male or female	90-95	1 mm^3	1 (after transplant)	5	1 g	—	—	1
Advanced 1st generation transplants for combination chemotherapy studies	Male or female	90-95	200 mg	±30 (after transplant)	6	> 4 g	± 60	—	2.5
Primary autochthonous tumors (see text)	Female								
Primary autochthonous tumors: adjuvant therapy after surgical enucleation‡	Female	—	1 g	1 (after enucleation, autochthonous tumors)	4-8 (varies with rest periods)	End point is + or − recurrence	± 60 (recurrent tumor deaths)	± 30	6.5§
Primary autochthonous tumors: pulmonary metastases	Female	—	Varies from <100 to >500 mg	—	Varies	4 g¶	—	—	Varies (about 3)¶

*Average total No. of drug(s) injections on a treatment schedule of 1 injection/wk.

†Data are for control animals.

‡Surgical enucleation (as opposed to "radical" excision) so as to deliberately leave behind microfoci of tumor cells for recurrence and as a target for the adjuvant therapy; there is usually a 90% tumor recurrence rate in the nonadjuvant-treated surgical controls.

§Delay in tumor recurrence rate can frequently be determined by 3 mos, but more certain end points (eg, cures) require 6.5 mos.

¶Each animal is killed when its calculated tumor weight = 4 g; presence of pulmonary metastases then determined by gross observation and/or lung bioassay.

Individual tumor measurements are obtained weekly with the aid of calipers and the approximate tumor weights (to about 15% error) are calculated by formula (8). It may be necessary to carry certain experiments longer to "split" the growth curves of two closely parallel combination chemotherapy groups into statistical significance; sometimes, the chemotherapeutic potency of a combination can be strong enough to bring about such a "split" earlier than the 2-2.5-month average duration for advanced transplant experiments.

Primary Autochthonous Tumors

Evaluation against spontaneous mammary tumors in their autochthonous hosts is the most rigorous test system. The therapeutic end point for determination of the effectiveness is either inhibition of tumor growth or transient tumor regression; no $CD8F_1$ tumors have been observed to undergo spontaneous regression. Cure has thus far been impossible to achieve by chemotherapy alone on large primary tumors. Hence, this most difficult methodology has been largely shelved in favor of evaluation by combined modality therapy; ie, in conjunction with the surgical reduction of primary tumors (134,135).

Primary Autochthonous Tumors: Adjuvant Therapy After Surgical Enucleation

Adjuvant therapy (chemotherapy and/or immunotherapy) can be evaluated in autochthonous mammary tumor-bearing $CD8F_1$ females after surgical enucleation of the primary tumor. "Enucleation" by surgery (simple stripping out of the tumor as opposed to "radical" excision) is performed in order to deliberately leave behind microfoci of tumor cells for recurrence as the target for the adjuvant therapy. The initial tumor weight at surgery averages approximately 1 g and there is usually a 90% tumor recurrence rate in the surgery controls. Surgery is performed on anesthetized mice under aseptic conditions, and the wounds are closed with Michel clips. The animals are separated into experimental groups of about 20 mice each by reference to a table of random numbers. The individual agents of combination chemotherapy have usually been given simultaneously beginning 1 day after surgery. They are administered once weekly thereafter with the average treatment course running for four to six weekly injections. The median day of appearance of tumor regrowth or recurrence is about 30 days after surgery, the median day of recurrent tumor deaths is about 60 days after surgery, and the average time after surgery for all controls to die is approximately 6.5 months. It is usually possible to discern a therapeutic end point (ie, a significant difference in tumor recurrence between experimental groups) some 3 months after surgery, but safer evaluation of cure rates can be made at 6 months, and the animals can be observed for lifetime cures. Throughout the course of the experiment, female mice can develop a "second" primary tumor in another breast, much as is the case in man. If the investigator does not wish to evaluate the effect of therapy on the "second" tumors, they can be removed by definitive excisional surgery and only the original sites of surgically enucleated tumors are followed for tumor recurrence. Another reason for radical surgery of second tumors is to prevent them from killing the host before the therapeutic effect on tumor recurrence of the original site of the surgically enucleated tumor has been evaluated.

Primary Autochthonous Tumors: Pulmonary Metastases Model

This spontaneous tumor-host system also provides a reproducible model to study therapeutic effects on spontaneously arising pulmonary metastases from autochthonous mammary adenocarcinomas. The incidence of metastases to the lungs correlates, in general, with the size of the primary tumor (8). The primary tumors may be left in situ or, if longer observation periods are desired, removed by definitive excisional surgery to prevent an earlier death from the large primary tumor. Our usual evaluation of activity against metastases consists of initiating treatment on primaries of less than 500 mg, and then continuing treatment until each tumor reaches a calculated weight of 4 g. At this point, each animal is killed and examined by gross observation, and/or by lung bioassay, for the presence of pulmonary metastases. Determination of drug effectiveness against mammary cancer metastases is based on comparison of the numbers of animals with positive lungs. Since the time required for tumors to reach 4 g is not uniform, the duration of the experiment varies, usually lasting about 3 months.

Single-Agent Chemotherapeutic Correlations Between Human Cancer, Human Breast Cancer, and the $CD8F_1$ Murine Mammary Cancer

Table 6 summarizes the chemotherapeutic correlations found between human cancer and $CD8F_1$ murine breast cancer with 12 compounds. These agents were selected by the DCT, NCI, as established clinically active (+) compounds against human cancer.[7] Testing these agents against the early $CD8F_1$ mammary tumor screening system identified 11 of the 12

[7]Carter S. List prepared by DCT, NCI, Bethesda, Md.

TABLE 6.—Single agent correlations between human breast cancer and $CD8F_1$ MMC

Clinically active agents*	Effect on MMC†
Cyclophosphamide	+
Adriamycin	+
Methotrexate	–
5-Fluorouracil	+
BCNU	+
Methyl-CCNU	+
CCNU	+
Melphalan	+
Mechlorethamine	+
Hexamethylmelamine	+
5-FUdR	+
ThioTEPA	+

*List submitted for testing by DCT, NCI.

†Chemotherapy once per week on 24-hr 1st generation $CD8F_1$ tumor transplant in male mice. + = Active; – = inactive.

compounds as active. By the same "early" transplant method, and in further confirmation of the value of this animal tumor model for predicting clinical effects, half (six of 12) of another DCT list of 12 compounds which were negative (–) against human cancer were found negative in the animal system, and the false positives then were found negative on further test against more stringent "advanced" mammary transplant systems. These overall results, particularly the 92% positive correlation with clinical results on human cancer (table 6), provide strong evidence that a solid tumor animal model that is therapeutically predictive for human solid cancer is available. As further indication that the $CD8F_1$ model may be generally useful as a predictive screen for human solid tumors, certain other compounds clinically useful against human solid cancers (eg, actinomycin D, 6-thioguanine, hexamethylmelamine, mitomycin C, and prednisone) have also been found active in this animal tumor model. Thus, the $CD8F_1$ tumor system appears generally responsive to agents of clinical interest against human solid tumors.

The 1975 "Summary Reports" of the DCT, NCI,[7] lists eight compounds as adequately evaluated and definitely active against human breast cancer. These eight drugs, with their activity against the $CD8F_1$ murine breast cancer in parentheses, are as follows: cyclophosphamide (+), adriamycin (+), 5-fluorouracil (+), methotrexate (-), melphalan (+), mitomycin C (+), vincristine (+), and vinblastine (+). (The latter two drugs are reported active against the $CD8F_1$ murine breast cancer when administered iv.)[8] Thus, only one of the list of eight compounds active as a single agent against human breast cancer is not similarly active against this murine breast cancer, an 88% positive correlation (seven of eight drugs) between the $CD8F_1$ murine breast cancer and human breast cancer.

Table 3 records methotrexate as the only failure in the therapeutic correlation between the mouse and human breast cancers. Clinical data on methotrexate activity against human breast cancer do not provide a breakdown as to the hormone dependence of the methotrexate-responding human breast cancers.[7] In this regard, it is of interest to note that the activity of methotrexate as a single agent against a hormone-sensitive rat mammary carcinoma appears conditioned by the drug's ability to induce an indirect endocrine effect; methotrexate has been demonstrated to increase serum estrogen levels and suppress prolactin.[9] From a chemotherapeutic point of view, the question of whether in this disease (breast cancer) methotrexate has both a specific antitumor effect and an indirect ability to affect endogenous hormone levels remains unanswered. If its activity in this disease is induced primarily indirectly, then it might be expected to be negative against the hormone-independent $CD8F_1$ mammary tumor. On the other hand, despite high concentrations in cell cultures of a murine spontaneous mammary tumor ($C3H \times O_2O$)methotrexate was without effect on this hormone-independent tumor both in vivo and in vitro, and the tumor was rated insensitive to methotrexate (10). However, on different murine spontaneous mammary carcinomas (A1st, DBA/2) methotrexate was ineffective in vitro but nevertheless inhibited in vivo tumor growth and extended median survival time (63). An attempt to unravel the problem of whether the direct and/or indirect antitumor effects have bearing on the refractory or sensitive state of hormone-independent tumors to methotrexate, or whether they may be synergistic or antagonistic to the chemotherapy of endocrine-responsive mammary cancer, is in progress.

The information available on the in vivo effects of single agents on various strains of spontaneous murine mammary tumors (208-210, 120, 67, 261, 49, 50, 131, 96, 202, 10, 66, 233) is difficult to compare owing to some differences in the methodology employed. However, comparison may be made where first generation transplants were employed, and, here, the $CD8F_1$ screening system appears to have somewhat greater predictive value. For example, certain important compounds (adriamycin) clinically active against both human breast cancer and the $CD8F_1$ tumor are reported as not effective against the C3H mammary tumor (202). Likewise, 5-fluo-

[8]Corbett T. Personal communication. Southern Research Institute, Birmingham, Ala.

[9]Bogden AE. Personal communication. Mason Research Institute, Worcester, Mass.

rouracil is active in the $CD8F_1$ tumor system but negative on other mammary transplants[10] and mammary tumor systems (67). The overall picture of therapeutically predictive correlations favors the $CD8F_1$ system. Also, this hybrid strain is healthier and hardier than most inbred strains, and is therefore the most suitable for combined modality research.

The utilization of the $CD8F_1$ mammary tumor system seems warranted both as a screen and as a more sophisticated research tool for identifying and eliciting the optimal activity of clinically useful anticancer agents.

Approximately 40 experimental tumor test systems are employed in the DR&DP screening program of the DCT, NCI. Since one or more animal tumor models are sensitive to nearly all clinically active agents, screening has yielded some useful drugs. However, these animal models are without demonstrated predictive correlation to the response of any specific type of human cancer, with the possible exception of the murine L1210 leukemia in relation to acute leukemia in man (98). Comparison of the L1210 activity data for the nine compounds established as clinically active against acute leukemia versus the $CD8F_1$ activity data for the DCT's list of 12 agents clinically active against human cancer, and DCT's clinically active list of eight drugs against human breast cancer, is of interest. The L1210 system-human leukemia correlation is 73% (98), whereas the $CD8F_1$ system-human cancer and human breast cancer identification of clinically useful compounds, is 92% and 88% respectively. Therefore, it seems valid to recommend the utilization of the $CD8F_1$ spontaneous mammary tumor system to expedite progress against both breast cancer (and solid cancers in general) in man.

Combination Chemotherapy Correlations Between Advanced Murine and Human Breast Cancer

Combination chemotherapy has been more successful than single-drug therapy against advanced cancer in both man and animals (57,58,82,93). This laboratory originally promulgated the combination chemotherapy of cancer (60,61,190-192,130,132,193-196,137,198-202,204), including a demonstration of effective results on the $CD8F_1$ murine spontaneous mammary carcinoma (143,84). The latter results, however, have not undergone clinical trial, and comparison with the laboratory data cannot be made.

More recently, however, we have screened in the "advanced" $CD8F_1$ first generation mammary tumor transplant system combinations of the single agents found individually active (table 6) against the "early" $CD8F_1$ mammary tumor transplant. The latter methodology provides a breast cancer model that is more resistant to chemotherapy than the "early" transplant model. It is also more relevant to clinical breast disease in that it is grossly apparent when treatment is started and is uniformly fatal if untreated. Two combinations of three agents have been found more effective against the advanced mammary tumor than any of their double combinations or the effectual dosage of their single agents: cyclophosphamide + adriamycin + 5-fluorouracil (CAF) and melphalan + adriamycin + 5-fluorouracil(PAF).[11]

Recent clinical trials against metastatic breast cancer have reported that the double combination of cyclophosphamide + adriamycin is more effective than either drug alone (126), and that the triple combination, CAF, is better than any of its component parts (38,53,210). Similarly, melphalan + 5-fluorouracil is reported more effective than either single agent (180).

The above positive correlations with combination chemotherapy against advanced breast cancer in both mouse and man recommend the $CD8F_1$ spontaneous murine mammary tumor system as predictive in identifying effective combination chemotherapy against advanced breast cancer in patients.

Combined Modality Therapy Correlations Between Primary Human and Murine Breast Cancers

The National Surgical Adjuvant Breast Project (NSABP) of the NCI's Breast Cancer Task Force has recently announced that prolonged melphalan administration as an adjuvant to mastectomy in the management of patients with primary breast cancer and pathologically positive axillary nodes has been demonstrated to be effective, particularly in those patients who are premenopausal (77). The NSABP previously reported higher 5- (78) and 10-year (77) survival times in premenopausal women having four or more positive axillary nodes treated with thio-TEPA versus a control receiving placebo. Overall acceptance of the clinical significance of these adjuvant findings has been minimized by the benefit ensuing to only one subset of patients (ie, premenopausal breast cancer patients). The contention is that the same effects could have resulted from hormonal therapy, and therefore the laboratory-proven principle that chemotherapy is more effective when the tumor mass is reduced at the start of treatment (197)

[10]Schabel FM, Jr, and Griswold DP. Personal communication. Southern Research Institute, Birmingham, Ala.

[11]Fugmann RA, Anderson JC, Stolfi RL, et al. Cancer Treat Rep. Submitted for publication.

has not been clinically validated. The suspicion is that the findings are related to a difference in the biologic behavior of the premenopausal and postmenopausal patients; namely, that the agents produced a suppression of ovarian activity (ie, a "chemotherapeutic oophorectomy") that resulted in the lack of significance in the postmenopausal group. However, an ongoing surgical adjuvant study, with triple combination chemotherapy after a mastectomy in breast cancer patients with positive axillary nodes, is currently demonstrating that adjuvant chemotherapy is clinically effective in both premenopausal and postmenopausal patients (33).[12]

The significance of positive nodes is that the 5-year survival rate is low in patients with positive axillary nodes found at the time of mastectomy (78); thus, positive nodes indicate a high likelihood that the disease has metastasized outside the local field of surgically removed tissue. Indeed, the presence of positive axillary nodes at the time of mastectomy has been shown to be a means of predicting with high probability that the appearance of previously unknown metastases will come early ("early" being defined as "within 2 years after surgery") (83). The additional therapeutic need is adjuvant chemotherapy. From the chemotherapist's point of view, the situation after removal of a primary human breast cancer with positive nodes is one of minimal residual malignant disease, a time when the number of cancer cells in the host is least and, therefore, when the chance of eradicating all the tumor cells with chemotherapy is greatest (197). The clinical validation of this basic principle of adjuvant chemotherapy is most important since current survival or end-result data indicate that two thirds of the people with solid cancers have metastases at the time the primary tumor is diagnosed (19).

The biologic evidence that provided this basic chemotherapeutic principle and the rationale for these successful surgical adjuvant chemotherapeutic treatments of human breast cancer were developed for the most part in our laboratory (131, 197,138), by employing the $CD8F_1$ spontaneous tumor model (133-135,139,143,144,141,84). The demonstrated applicability of this principle (ie, the smaller the number of tumor cells, the greater the chemotherapeutic effect) in both the $CD8F_1$ solid tumor animal model and human breast cancer is an additional reason to accept the $CD8F_1$ solid tumor animal model as therapeutically predictive for human breast cancer (and possibly other human solid cancers).

[12]Bonadonna G. Personal communication. Istituto Nazionale Tumori, Milan, Italy.

IMMUNITY, IMMUNOTHERAPY, CHEMOIMMUNOTHERAPY—THE $CD8F_1$ MAMMARY TUMOR SYSTEM AND HUMAN BREAST CANCER

The major usefulness of the $CD8F_1$ model system of spontaneous, autochthonous murine breast cancer concerns its immunologic relevance to the human situation, particularly in its interaction with chemotherapy. As mentioned earlier, the contribution of immunity to the therapeutic effects of anticancer agents is critical at the level of cure (140,136).

The key role of host immunity in the ultimate effectiveness of the chemotherapeutic response was demonstrated with the $CD8F_1$ model system. First, by indirect evidence it was shown that the chemotherapeutic response of the murine breast cancer could be both nullified by the administration of an immunosuppressive regimen of cortisone (135, 139,84) and enhanced by the administration of a nonspecific immunostimulant, zymosan (144,143,84). Subsequently, direct evidence of the beneficial participation of host immunity in therapeutically induced tumor cures was obtained by measurement of immunologic parameters (eg, cell-mediated immunity and humoral factors including blocking activity) in the host during treatment (219). In these studies the influence of tumor size on host immunity was firmly established, and a correlation was demonstrated between a favorable immune setting and the attainment of cure with a particular combination of drugs.

The tumor burden was seen to be the major factor controlling therapeutic cure. Apparently, the capacity of cancer chemotherapy *alone* to kill the last cancer cell in the face of an appreciable number of tumor cells is severely limited by its "built-in" first order kinetics (208,249) and inherent drug toxicity (140). With the assistance of surgical removal of the gross tumor, however, adjuvant chemotherapy can further reduce the tumor load to a level low enough to be overcome by innate or augmented host immunity (219).

There is a ceiling (which is relatively low) on the maximum tumor burden approachable by immunotherapy (136,140). Chemotherapy can attack a large tumor load but can not eliminate all the tumor cells; immunotherapy is ineffectual on large tumor loads but can eliminate small numbers of cells. Obviously, chemotherapy and immunotherapy can complement each other to effect cure. Unfortunately, this neat but complex solution is further complicated by the additional limitation that chemotherapy usually causes immunosuppression (136,140,22), and thwarts a therapeutic complementation by host immunity. However, it is possible to measure the immunologic parameters adversely affected by the

immunosuppressive activity of anticancer agents on the $CD8F_1$ tumor-host system (85) and to ultimately utilize this information to plan therapeutic schedules to minimize these untoward effects.

The demonstration that nonspecific immunotherapy (eg, zymosan) can augment a chemotherapeutic effect on the $CD8F_1$ spontaneous tumor (143,144,84) correlates with similar studies in human breast cancer. It has recently been shown that after chemotherapeutic reduction of tumor load, nonspecific immunotherapy (eg, bacillus Calmette-Guérin) can enhance the chemotherapeutic response of breast cancer in women.[13]

The general recognition that (a) the size of the tumor cell burden is of signal importance in the chemoimmunotherapeutic treatment of cancer in man, (*b*) some help from host resistance is necessary for chemotherapeutic cure, and (*c*) an understanding of the immunosuppressive effects of chemotherapeutic agents on the host-autochthonous tumor relation may facilitate therapeutic management, must be considered in any examination of spontaneous versus transplantable solid tumors (eg, Ehrlich ascites, Sarcoma 180, Carcinoma 755, etc) as predictive animal models. Such transplantable tumors in their allogeneic systems can lead to the development of strong transplantation immunity that unduly participates in the killing of the tumor cells and leads to optimistic evaluation of drug effects which will probably not be valid in human or animal autochthonous tumor systems. Not only must the chemotherapist be aware of the contribution of immune defenses to the overall therapeutic effect, but the immunologic guidelines supplied by the laboratory must have validity to the autochthonous tumor problem. For example, complement from different animal species, when used with the same samples of antibody, can effect quantitative and even qualitative differences in immune lysis assayed in vitro.[14] Such data illuminate and underscore the importance of using antibody, complement, and target tumor cells from a single autochthonous host-tumor system to obtain data for extrapolation to in vivo phenomena. The interaction of drug, host, and tumor factors is responsible for the net result obtained in any tumor-host system. However, in the *autochthonous* tumor system the interrelationships are more complex and subtle than in the transplantable tumor systems, and yet they are essential to cure. Thus, with regard to immunologic-chemotherapeutic interrelationships effecting cure, the information accumulated on the $CD8F_1$ model system of spontaneous, autochthonous murine breast cancer would appear to offer assurance of predictive value for effective translation against human breast cancer and, probably, other autochthonous tumors in man as well.

$CD8F_1$ Tumor Metastasis Model and Human Breast Cancer

The majority of human solid cancers (19), including breast cancer (78), have metastases when first presented for treatment. And metastases are clearly the problem thwarting the cure of most solid tumors, since primary treatments with surgery and/or radiotherapy are reasonably effective locally. A valid solid tumor metastases model in an autochthonous host is necessary because immunologic factors have been found paramount in the control of the dissemination of tumor cells and their growth as metastases.

Highly antigenic tumors do not metastasize but weakly antigenic ones do (118); nonmetastasizing tumors can be made to metastasize in immunologically depressed hosts (1,55); circulating humoral factors can inhibit pulmonary metastases (176); and drug-induced immunosuppression can elicit metastases in animals and men (256,14,68,98,169,244). There is a good correlation between depressed immunocompetence (from whatever cause) and tumor recurrence or metastases in patients (170,4,212,70, 228). In the presence of large tumor loads, the host's tumor-specific immunity, paradoxically developing concomitantly with the growing tumor, may be replaced by a state of immunologic unresponsiveness (248,122,13,124,235,238,226,52,15,219,233) which can be correlated with increased metastases (226,8). This depression of immunity is caused by increased shedding of the tumor antigen associated with progressive tumor growth (203,48,12), resulting in the formation of antigen-antibody complexes which function as blocking factors to interfere with effective host immunity (41,207). Removal of the large primary tumor mass causes the disappearance of the blocking factors from serum and the restoration of host immunity (13,248,219,122,220); however, the tumor metastases established by then remain largely unaffected. Thus, the development of metastases is influenced by the immunologic competence of the host (148,39,234,239,176). Indeed, immunologic impairment has been shown to have a more striking effect on metastatic growth and development than on the primary tumor growth (39).

From these facts it seems clear that advances in the clinical therapy of metastases will require knowledge of the interrelations between the host, the autochthonous solid primary tumor, the tumor metastases, and the drug treatment regimen. From the chemotherapist's viewpoint, it is necessary to

[13] See footnote 6.

[14] Grant CK. Complement origin determines lytic activity of antibodies to nucleated target cells. Comparison of common complement sources. Transplantation. Submitted for publication.

recognize that growth characteristics of metastases can be different from the parent primary tumor (225), and small tumor cell populations in distant metastases have been demonstrated to have a higher growth fraction than those in the parent primary tumor (205). Therefore, it is not surprising that drug response of grossly visible tumor masses may not predict for the qualitative or quantitative sensitivity of micrometastases (182).

Recent research in the $CD8F_1$ tumor metastases system exemplifies many of the problems. It was found that the adjuvant triple combination chemotherapy of PAF given after surgical removal of spontaneous breast tumors in autochthonous hosts initially appeared to produce cures. However, this exciting result was more apparent than real, in that the "cure" rate rapidly declined after 4 months to equal that of the surgical controls.[15] The final chemotherapeutic effect was only that of delayed tumor recurrence. The animals were killed at tumor recurrence and examined for lung metastases. The incidence of pulmonary metastases from these spontaneous tumors was found to be equivalent in the control and PAF-treated animals. Although PAF produced an impressive inhibitory effect on the recurrence of the primary tumor, this particular drug regimen was entirely ineffective in curing even small pulmonary metastases.[15]

An explanation for these findings was afforded by the measurement of immunologic parameters in PAF-treated $CD8F_1$ animals, revealing that this triple drug combination schedule produced marked immunosuppression.[15] It is of interest that immunologic impairment can have a more striking effect on the growth of metastases than on that of the primary tumor (39). Further, it is necessary to recognize that chemotherapeutic cure is dependent on the synergistic interaction of immune mechanisms (133,139,219,84). Chemotherapy which depresses the host's immunologic defenses may be harmful if its immunosuppressive activity outweighs its tumor destructive effects (eg, can increase metastases), or it may be limited in beneficial effects (ie, tumor inhibitory rather than curative), or it may be beneficial if it is scheduled so as to favor recovery of host immune cells over that of the tumor cells (ie, tumor destruction > immunosuppressive activity). In the cited example of the immunosuppressive combination of PAF, only tumor inhibition ensued whereas other less immunosuppressive combinations of drugs and their schedules have effected cures in the same surgical adjuvant chemotherapy situation (219,85,142). Knowledge that one particular drug combination is inhibitory at best, whereas another is capable of cure, is obviously significant to any consideration of a trial in man. It takes considerable time, effort, and money to pursue a clinical trial, and the number of cancer patients available, suitable, and willing to cooperate in such therapeutic evaluation tests is limited. A solid tumor animal model that is therapeutically predictive for human breast cancer would serve a critical role.

Increasing numbers of drug combinations are being suggested for clinical evaluation as adjuvant therapy against solid tumors. The clinical evidence supporting the recommendation is invariably that of marked tumor inhibition or regression against advanced human disease. Yet the end point of regression is not the same as cure. Regression is dependent upon killing many cancer cells; cure requires destroying all cancer cells. Regression can be effected by chemotherapy, particularly combination chemotherapy, but the last few residual cancer cells will not be eliminated in the presence of marked, or enough, concomitantly induced drug-immunosuppression. Unfortunately, suppression of the immune response by combination chemotherapy is common (22). Therapeutic effort aimed at clinical cure requires more precise guidance in the optimal use of these drugs. Fortunately, an optimal therapeutic schedule, or even a slight change in choice of one or more agents in the combination, can avoid undue immunosuppression and thereby allow cure (142, 219,85). The identification of clinically useful drugs, or drug combinations, is not enough. Otherwise, there is the likelihood that the selection of chemotherapy for clinical evaluation on the sole basis of drug-induced transient tumor regression in advanced disease will waste time, effort, funds, and patient lives. Such trials are needlessly blind if there is available a therapeutically predictive solid tumor animal model that can effectively discriminate between several drug combinations proposed for clinical trial as to their varying capacities to induce cure (as opposed to merely causing prolonged periods of tumor regression and inhibition). Cure, as opposed to delay in tumor recurrence, is the goal. Additional important considerations are those of time and money, particularly if the animal model can accomplish such evaluation in 6 months, compared with clinical trials for the same purpose that require from 18 months to 2 years.

The accumulated data that have been presented compel the conclusion that the $CD8F_1$ spontaneous, autochthonous murine mammary tumor is a therapeutically predictive animal model for human breast cancer. Since general principles appear to be broadly valid in biology, it is likely that this solid tumor animal model will provide therapeutic guidelines which are applicable to other human solid tumors as well.

[15] See footnote 11.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

CAF: cyclophosphamide, adriamycin, and 5-fluorouracil

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

5-FUDR: NSC-27640; uridine, 5-fluoro-2′-deoxy-

Hexamethylmelamine: NSC-13875; CAS reg. No. 645-05-6

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-; L-phenylalanine mustard

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl) methyl]methylamino]benzoyl]-

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

Mitomycin C: NSC-26980; CAS reg. No. 50-07-7

PAF: melphalan, adriamycin, and 5-fluorouracil

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,-11,20-trione, 17,21-dihydroxy-

6-Thioguanine: NSC-752; CAS reg. No. 1832-72-0; purine-6-thiol, 2-amino-

ThioTEPA: NSC-6396; CAS reg. No. 52-24-4; phosphine sulfide, tris(1-aziridinyl)-

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ADDENDUM

Since this report was accepted for publication, additional research has shown an earlier finding—ie, that methotrexate is the only one of eight agents clinically active against human breast cancer that is inactive against $CD8F_1$ murine breast cancer—to be erroneous. To the contrary, methotrexate *is* active. This earlier inaccurate statement probably resulted because methotrexate is a toxic agent with a very narrow therapeutic index in the $CD8F_1$ situation. Of nine experiments with methotrexate alone, five could not be evaluated due to toxic effects (ie, either excessive mortality or severe weight loss precluded proper evaluation of antitumor effect). The remaining four experiments with methotrexate as a single agent were evaluable and revealed significant antitumor activity of methotrexate. Subsequently, in three evaluable experiments where citrovorum factor rescue successfully counteracted the toxicity of methotrexate there was also significant antitumor activity. Other experiments evaluating therapy with the combination of methotrexate and 5-fluorouracil documented a therapeutic synergism against this murine breast cancer (in over 40%) that was both schedule- and sequence-dependent (ie, methotrexate had to be given 1 hour before 5-fluorouracil).

These findings show the activity of methotrexate against the $CD8F_1$ murine breast cancer. Thus, all of the eight compounds listed by the NCI as clinically active as single agents against human breast cancer are active against this murine breast cancer, a 100% positive correlation in chemotherapeutic response between the $CD8F_1$ murine breast cancer and human breast cancer.

Growth Characteristics and Chemotherapeutic Response of Intravenously Implanted Lewis Lung Carcinoma

Artemio A. Ovejera,[1,2] Randall K. Johnson, [1,3] and Abraham Goldin[1]

SUMMARY

Iv implantation of Lewis lung carcinoma gave rise preferentially to tumor growth in the lungs with very little involvement of other organs. The pulmonary tumor had a mass doubling time of 1.6 days when the tumor was 50 mg and 6.0 days during the terminal stages of the disease. The range of death in untreated $B6D2F_1$ mice inoculated iv with 1×10^5 viable Lewis lung tumor cells was relatively narrow; in 19 experiments, the median survival time of control groups ranged from 19.0 to 25.5 days. The chemotherapeutic response of early (treatment started on Day 1) or advanced (treatment started on Day 14) iv Lewis lung tumor to a spectrum of clinically active antitumor agents was evaluated. Cyclophosphamide and BCNU were curative on early treatment and increased lifespan on treatment of advanced tumor. Against early tumor, adriamycin was considerably more effective when administered iv as compared to ip; against advanced tumor, iv administered adriamycin exhibited marginal activity whereas the drug administered ip was inactive. 5-FU and 5-FUdR exhibited significant activity against both early and advanced tumor. Melphalan, mechlorethamine, bleomycin, and cytosine arabinoside were active against early tumor only. Actinomycin D, hexamethylmelamine, thioTEPA, methotrexate, vinblastine, and vincristine were inactive at tolerated doses. Cyclophosphamide and BCNU were therapeutically synergistic in combination against advanced tumor. Iv Lewis lung appears to be a useful solid tumor model for the evaluation of potential antitumor agents.

[Cancer Chemother Rep Part 2, vol 5:111-125, 1975]

Lewis lung carcinoma is a highly malignant anaplastic carcinoma which originated spontaneously in the lungs of a C57BL/6 mouse in 1951 (1) and has since been maintained by serial transplantation. This tumor, implanted either im or sc, has been used for detailed drug evaluation in a number of laboratories. Lewis lung carcinoma implanted im or sc may have limited predictive value because it is refractory to most compounds with established activity against human cancer. It is quite sensitive to cyclophosphamide and the nitrosoureas (2-4) and shows limited response to 5-FUdR (2,4,5) and to bleomycin (4-6).

Lewis lung carcinoma metastasizes to the lungs in the early stages of tumor growth at the site of implantation (3,7,8). As a metastasis model, Lewis lung carcinoma has been used both to differentiate between specific effects of drugs on metastases as opposed to effects on primary tumor growth (7-10) and to investigate principles of surgical adjuvant chemotherapy (7,8,11).

Simpson-Herren et al have studied the kinetics of primary sc Lewis lung carcinoma (12) as well as the kinetics of spontaneous lung metastases (12,13) and "artificial" pulmonary metastases induced by iv inoculation of tumor cells (13). They observed that the proportion of proliferating tumor cells (based on the thymidine index) decreased with tumor age for the primary and spontaneous metastases whereas for "artificial" metastases, it remained unchanged through Day 22. At advanced stages of tumor growth, cell cycle time was shorter for spontaneous and "artificial" metastases (14-16 hours) as compared to that of the primary sc tumor (24 hours). Volume doubling time was longer for the primary tumor and "artificial" metastases (8-10 days) than for the spontaneous metastases (2 days).

This investigation assesses the growth characteristics of lung tumors resulting from iv implantation

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of Lewis lung carcinoma. The chemotherapeutic response of mice bearing early or advanced tumor to a varied group of clinically active antitumor drugs was evaluated to ascertain the usefulness of this tumor system as a lung tumor model for the selection of potential antineoplastic agents.

MATERIALS AND METHODS

The Lewis lung carcinoma was maintained by biweekly transplantation of a suspension of approximately 1×10^6 viable tumor cells in 0.1 ml im into the hind leg or sc into the inguinal region of C57BL/6 mice. Tumor inoculum was prepared aseptically by pressing nonnecrotic tumor tissues through a 40-gauge metal sieve and suspending the material in Hank's balanced salt solution. Clumps of cells and tissue fragments were removed by passing the suspension through a double layer of sterile gauze pad before making the cell count and the final dilution of tumor inoculum. Cell viability as determined by trypan blue exclusion was about 15%.

Test animals were male $B6D2F_1$ (C57BL/6 female × DBA/2 male) mice weighing 18-24 g and 6-7 weeks of age. Each mouse was inoculated via a tail vein with 0.2 ml of tumor suspension containing 1×10^5 viable cells.

For studies of the growth of Lewis lung tumor in the lungs after iv implantation, groups of ten mice were selected randomly from a large group of tumor-bearing mice starting on Day 8 and every 2 days thereafter until Day 24. The lungs of five mice were excised, blotted with gauze, and weighed. Macroscopic tumor nodules on the lung surfaces of the other five mice were counted using the procedure of Wexler (14).

Bioassays were performed to determine the distribution of viable tumor in the different organs (lungs, liver, spleen, kidneys, and brain) of mice 15 and 21 days after iv implantation of 1×10^5 Lewis lung tumor cells. The lungs of three donor mice were excised, cut into small pieces, and put into a 6-ml syringe. Hank's balanced salt solution was added to make a 4.0-ml volume, and the mixture was passed several times through a 19-21-gauge needle. A similar procedure was used for each organ. Each organ suspension was injected im into ten mice (0.1 ml/mouse) and sc into another ten mice (0.2 ml/mouse). The recipient mice were observed for tumor growth and survival.

A study was conducted to evaluate the chemotherapeutic response of early and advanced iv implanted Lewis lung carcinomas to 15 clinically active antitumor drugs. Treatment for early tumor began 1 day after tumor inoculation; for advanced tumor, treatment began on Day 14. Drug solutions were administered ip, unless otherwise specified, in a volume of 0.01 ml/g of body weight. Median survival time (MST, median day of death) relative to untreated control groups was used as the criterion for evaluation of drug activity.

RESULTS

Growth of Lewis Lung Tumor in $B6D2F_1$ Mice After Iv Implantation of 1×10^5 Cells

The average weight of the lungs and the average number of tumor colonies in the lungs at different time periods in mice inoculated iv with Lewis lung tumor are presented in table 1. The weight of tumorous lungs increased steadily with time. However, there was no significant change in the number of tumor nodules in the lungs relative to time after tumor inoculation. The average number of nodules ranged from 13 to 25.

Figure 1 shows the weight of the lung tumor plotted against the time after implantation. The weight of the lung tumor was obtained by subtracting the weight of normal lungs from the weight of tumored lungs. Eight days after implantation, tumor in the lungs averaged about 8 mg. By Day 14 tumor weight was about 100 mg and in the terminal stage of the disease tumor in the lungs weighed over 500 mg. Mass doubling time as estimated from the growth curve was 1.6 days when the tumor was 50 mg and 6.0 days during the terminal stages of the disease.

The lung was the only organ in which macroscopic tumor growth was evident. The tumor nodules became visible as pinpoint lesions 6-8 days after implantation and grew progressively to as large as 8 mm in diameter. At the time of death (about 21 days), the tumor nodules appeared confluent and enveloped practically the entire lung surface.

Bioassays of different organs (table 2) revealed that the lung was the major site of tumor proliferation. Mice injected im and sc with tumor homogenate prepared from lungs of mice bearing 15-day iv Lewis lung carcinoma had MSTs of 25.0 and 37.0 days, respectively. Similar results were obtained for the Day-21 lungs. Bioassay of other organs at 15 days after implantation did not detect the presence of viable tumor. At 21 days after implantation, there was minimal involvement of the other organs with the exception of the kidney.

Survival of Mice Inoculated Iv With 1×10^5 Lewis Lung Carcinoma

Survival data of 307 untreated control mice from 19 experiments were pooled and the death distribution and cumulative survival curve are shown

graphically in figure 2. Of the 307 untreated mice, two mice survived beyond 90 days with no gross evidence of tumor growth in the lungs. The arithmetic mean of the MSTs of control groups from 19 experiments was 21.5 ± 1.75 (standard deviation [SD]) days with a range of medians in individual experiments of 19.0-25.5 days (table 3). Mayo et al(4)reported a 21.0-day MST for mice inoculated iv with 1×10^6 Lewis lung tumor cells. For sc Lewis lung tumor, Mayo (3) reported a pooled MST of 25.5 days with a range of medians of 21.0-32.0 days for 205 mice from 18 experiments. The range of deaths of individual animals in the present study was 11-45 days.

TABLE 1.—Average weight of lungs and average No. of tumor nodules in lungs of mice at different time periods after iv implantation of 1×10^5 Lewis lung carcinoma cells*

Day after implantation	Average weight of lungs (mg)	Average No. of tumor nodules
0 (control)	149.2 ± 12.1	—
8	157.0 ± 13.6	18.8 ± 14.0
10	166.6 ± 7.0	12.8 ± 12.2
12	173.4 ± 11.5	24.0 ± 15.4
14	251.4 ± 24.0	18.8 ± 7.2
16	330.6 ± 36.5	23.6 ± 9.0
18	496.4 ± 177.2	25.2 ± 10.6
20	642.0 ± 188.8	14.2 ± 8.1
22	711.8 ± 276.1	14.4 ± 7.4
24	932.4 ± 154.8	17.8 ± 8.4

*Each value represents the mean of 5 observations.

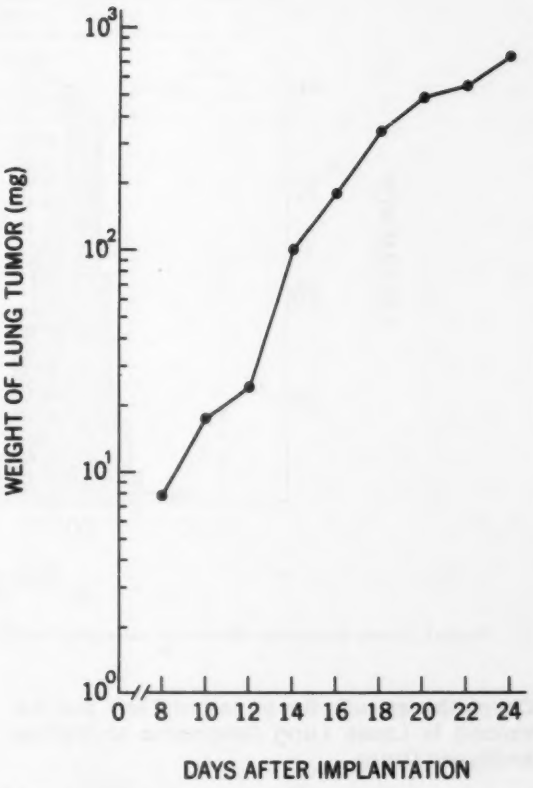

FIGURE 1.—Tumor growth in the lungs of mice after iv implantation of 1×10^5 Lewis lung carcinoma tumor cells.

TABLE 2.—Organ bioassay of mice bearing 15- and 21-day iv Lewis lung tumors

Donor organ	Recipients' inoculation site	Day 15 assay: No. of mice that died*/No. inoculated	Day 15 assay: MST (days)	Day 15 assay: Range of death (days)	Day 21 assay: No. of mice that died*/No. inoculated	Day 21 assay: MST (days)	Day 21 assay: Range or day of death (days)
Lung	Im	10/10	25.0	18-37	10/10	24.5	19-31
	Sc	9/9	37.0	32-41	8/9	37.0	27-50
Liver	Im	0/10	—	—	1/10	—	43
	Sc	0/10	—	—	0/10	—	—
Spleen	Im	0/10	—	—	1/10	—	39
	Sc	0/10	—	—	2/10	—	41, 58
Kidney	Im	0/10	—	—	9/10	53.0	38-58
	Sc	0/10	—	—	1/10	—	—
Brain	Im	0/10	—	—	1/10	—	—
	Sc	0/10	—	—	0/10	—	—

*All animals that died had tumor; those that were alive until the end of the experiment (65 days) had no apparent tumor growth at implantation site.

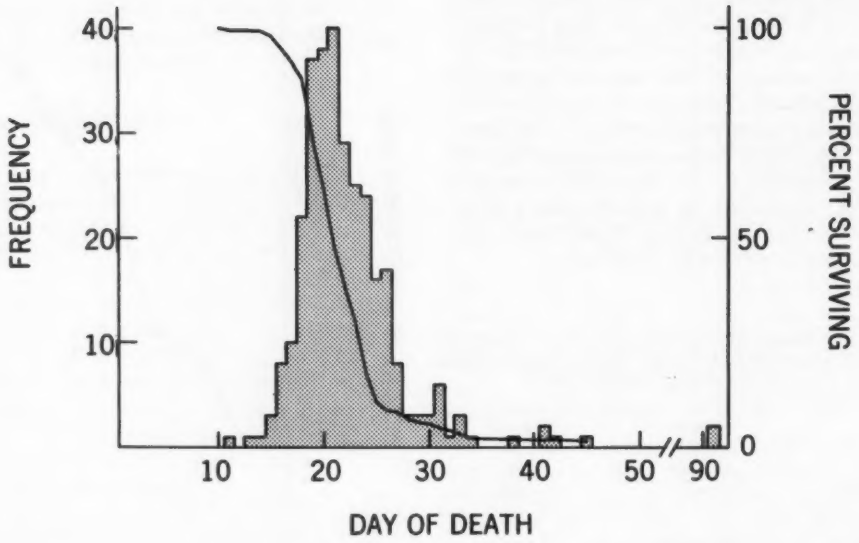

FIGURE 2.—Death distribution and survival curve of 307 B6D2F$_1$ mice implanted iv with 1×10^5 Lewis lung carcinoma tumor cells.

Chemotherapeutic Response of Early and Advanced iv Lewis Lung Carcinoma to Various Antitumor Drugs

The results of chemotherapeutic trials with early and advanced iv Lewis lung carcinoma are presented in figures 3-13. Cyclophosphamide (fig 3) was curative against early tumor and increased the lifespan on treatment of advanced tumor. A single injection of 133 mg/kg of the drug on Day 1 afforded cure[4] for seven of eight treated mice. Treatment of early tumor with 200 mg/kg of cyclophosphamide increased the median lifespan by 179% and gave three of eight 90-day survivors. Treatment of advanced tumor (Day 14) with 200 or 300 mg/kg of cyclophosphamide yielded an increase in lifespan (ILS) of 40% and 70%, respectively.

A similar degree of activity was shown for BCNU (fig 4). Treatment with 30 mg/kg of BCNU on Day 1 was curative for six of eight mice and a dose of 20 mg/kg increased lifespan by 138% and resulted in two of seven 90-day survivors. Administration of BCNU at 40 mg/kg against advanced tumor elicited an ILS of 52%.

Adriamycin was more effective against early iv Lewis lung tumor when administered iv than ip as can be seen in figures 5 and 6. The maximum ILS brought about by iv administration of adriamycin (14 mg/kg) on Day 1 was 155% compared to 45% for ip drug administration (optimal dose, 9.8 mg/kg). The dose of 14 mg/kg given ip was toxic. Marginal activity (26% ILS) was obtained with iv treatment of advanced tumor, whereas treatment by the ip route was totally ineffective against advanced tumor.

The activity of 5-fluorouracil (5-FU) against iv Lewis lung carcinoma is shown in figures 7 and 8. Treatment on Days 1, 5, and 9 (fig 7) with 44 mg/kg produced an MST of 41.5 days (98% ILS). Extended treatment (every 4 days on Days 1, 5, 9, 13, 17, and 21) proved to be less effective than treatment on Days 1, 5, and 9. Against advanced tumor, 67 mg/kg on Days 14, 18, and 22 increased lifespan by 36% over the controls. The optimal dose of 5-FU (20 mg/kg) given on a daily treatment schedule for 9 days increased lifespan by 71% against early tumor and 33% against advanced tumor (fig 8). Extended daily treatment (Days 1-22) resulted in 95% ILS at the optimal dose of 14 mg/kg/day. Extended daily treatment at doses of 20 mg/kg/day and higher was toxic.

The antimetabolite, 5-FUdR, exhibited activity against both early and advanced iv Lewis lung tumor (fig 9). Treatment on Days 1, 5, and 9 with 210 or 300 mg/kg resulted in 33% ILS. Against advanced tumor, the dose of 300 mg/kg given on Days 14, 18,

[4] "Cure" is defined as survival to 90 days which is the observation period for the experiment.

TABLE 3.—MSTs of 19 groups of control mice implanted iv with 1×10^5 Lewis lung carcinoma cells

No. of animals	MST (days)	Range of death (days)	No. of 90-day survivors
16	21.5	16-26	0
16	20.0	11-38	0
15	20.0	16-26	0
16	21.0	14-26	0
10	20.5	18-25	0
16	19.0	17-27	0
16	20.0	17-26	0
16	19.5	13-22	0
16	21.5	18-26	0
16	22.0	18-31	1
16	25.0	21-42	0
16	24.0	19-41	1
16	22.5	18-28	0
16	25.5	16-45	0
10	22.5	20-34	0
16	21.0	17-29	0
16	21.0	15-31	0
24	21.0	16-41	0
24	21.5	19-31	0
307	21.5 (Mean)	11-45	2

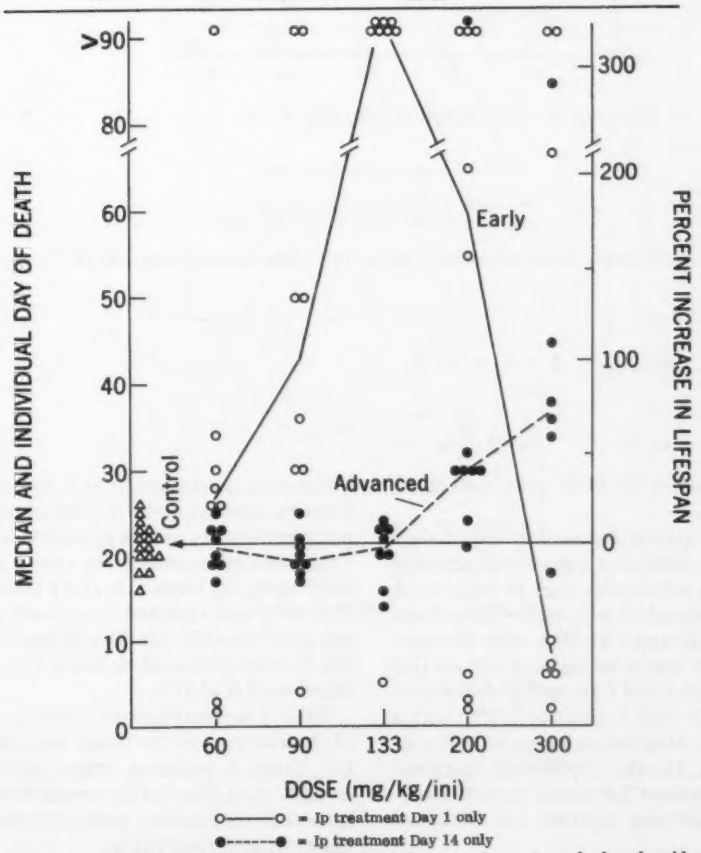

FIGURE 3.—Chemotherapeutic response of iv Lewis lung carcinoma to cyclophosphamide.

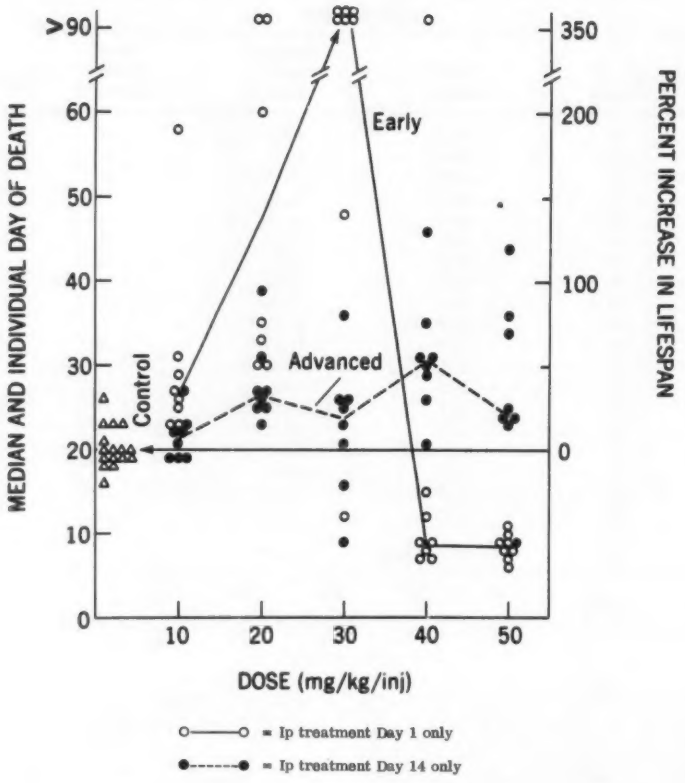

FIGURE 4.—Chemotherapeutic response of iv Lewis lung carcinoma to BCNU.

and 22 increased lifespan by 60% over untreated controls.

Two clinically active alkylating agents, melphalan and mechlorethamine, exhibited significant activity against iv Lewis lung carcinoma (figs 10 and 11). A dose of 7 mg/kg of melphalan administered on Days 1, 5, and 9 increased lifespan by 56% over the controls and a dose of 4.9 mg/kg brought about an ILS of 33%. Treatment with 2 and 1.33 mg/kg of mechlorethamine on Days 1, 5, and 9 effected a 37% and a 26% ILS, respectively. Melphalan, given at a dose of 10 mg/kg on Days 14, 18, and 22, showed marginal activity (25% ILS) against advanced tumor. Mechlorethamine had no activity against the advanced disease.

Cytosine arabinoside and bleomycin manifested activity against early iv Lewis lung tumor but were not significantly active against the advanced tumor. Cytosine arabinoside given over a dose range of 686-1400 mg/kg on Days 1, 5, and 9 increased lifespan by 31%-40% and resulted in a small proportion of 90-day survivors (fig 12). The optimal dose of bleomycin (fig 13) was 40 mg/kg on Days 1, 5, and 9 which produced an ILS of 32%.

Table 4 summarizes the activity of the drugs tested. In addition to the drugs described above (figs 3-13), table 4 includes drugs which were inactive (≤ 25% ILS). The latter drugs were actinomycin D, hexamethylmelamine, methotrexate, thioTEPA, vinblastine, and vincristine.

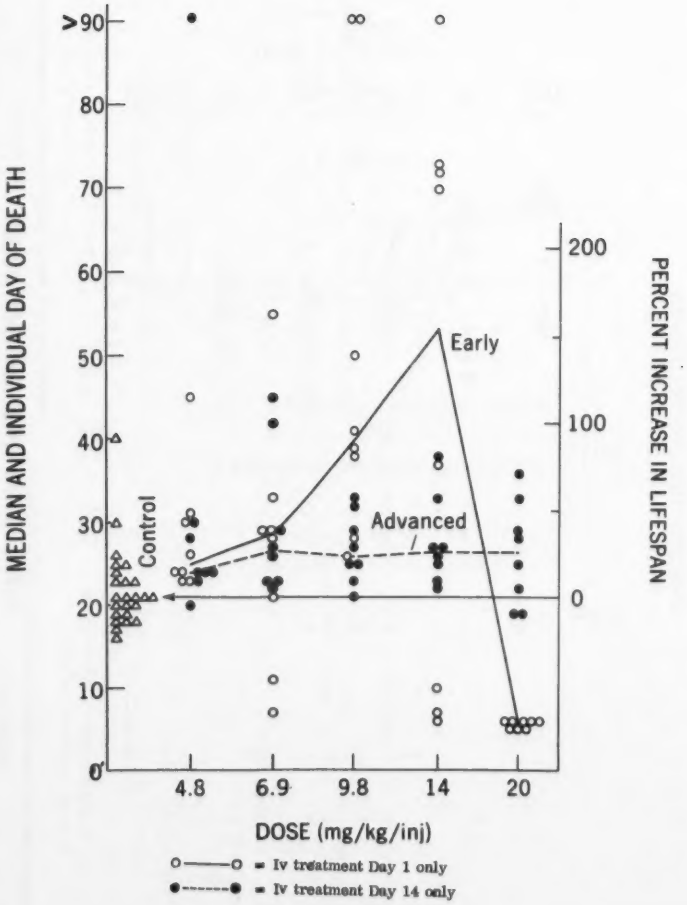

FIGURE 5.—Chemotherapeutic response of iv Lewis lung carcinoma to adriamycin.

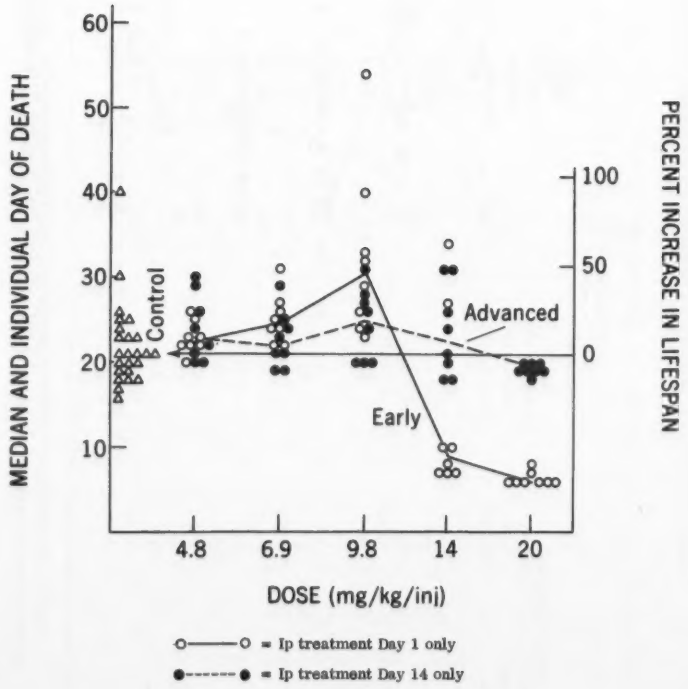

FIGURE 6.—Chemotherapeutic response of iv Lewis lung carcinoma to adriamycin.

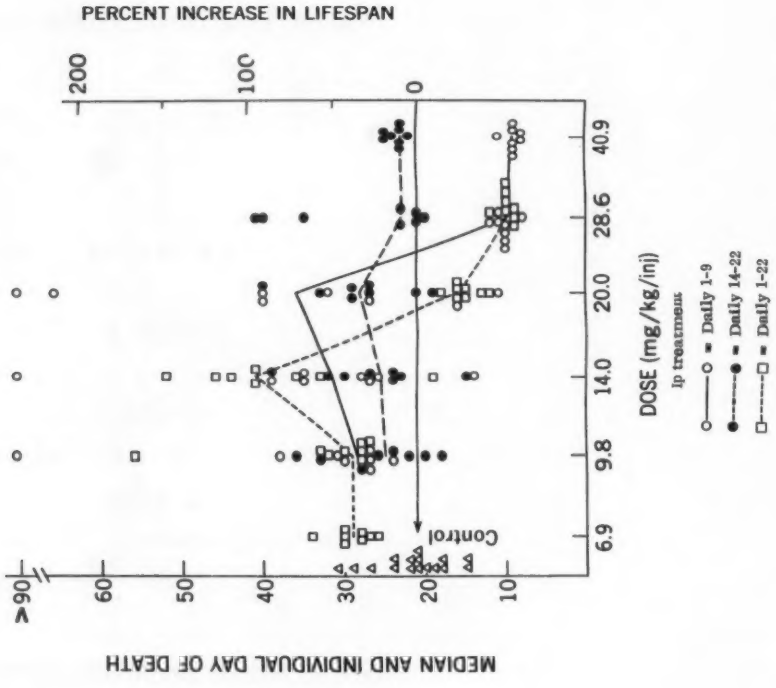

FIGURE 8.—Chemotherapeutic response of iv Lewis lung carcinoma to 5-FU.

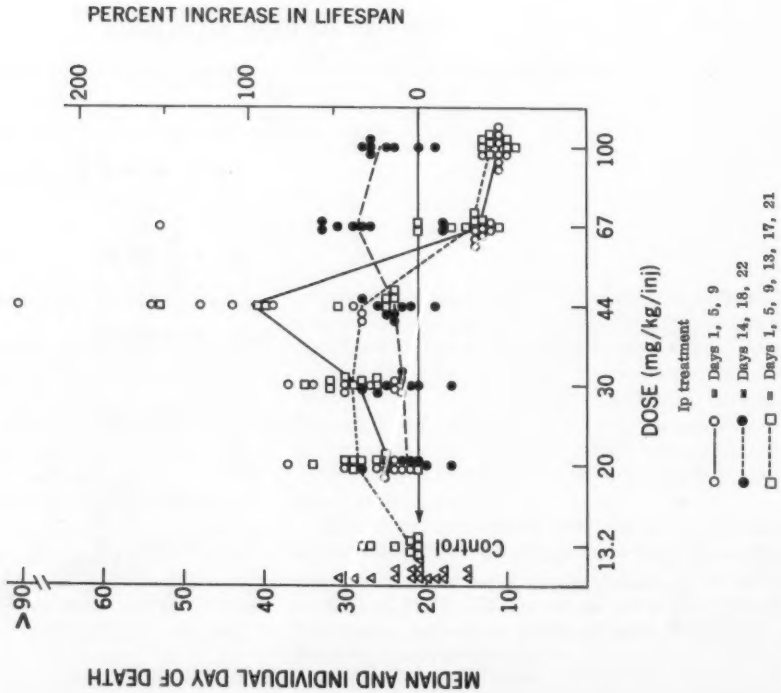

FIGURE 7.—Chemotherapeutic response of iv Lewis lung carcinoma to 5-FU.

TABLE 4.—Activity of clinical anticancer agents against iv implanted Lewis lung carcinoma

Drug	Dose range (mg/kg/ injection)	Early tumor*		Advanced tumor†	
		Dose (mg/kg)	Maximum %ILS (No. of 90-day survivors/ No. treated)	Dose (mg/kg)	Maximum %ILS
Cyclophosphamide	60-300	133	>319(7/8)	300	72
BCNU	10-50	30	>350(6/8)	40	52
Adriamycin (iv)	4.8-20	14	155(1/8)	6.9-20	26
Adriamycin (ip)	4.8-20	9.8	45	9.8	19
5-FU	20-100	44	98(1/8)	67	36
5-FUdR	72-300	210,300	33	300	60
Melphalan	3.4-20.4	7	56	10	26
Mechlorethamine	0.59-3	2	37	0.88,3	7
Bleomycin	12-60	40	32	60	15
Cytosine arabinoside	480-2000	1400	40(1/8)	780	19
Actinomycin D	0.01875-0.6	0.15	14	0.15	18
Hexamethylmelamine	59-675	300	5	133	9
Methotrexate	2.5-30	20	17	—	0
ThioTEPA	2.6-20	—	0	2.6	22
Vinblastine	0.125-2	0.125	4	2	7
Vincristine	0.125-2	1	12	1	12

*Treatment on Days 1,5, and 9 except for cyclophosphamide, BCNU, and adriamycin which were given on Day 1 only.
†Treatment on Days 14, 18, and 22 except for cyclophosphamide, BCNU, and adriamycin which were given on Day 14 only.

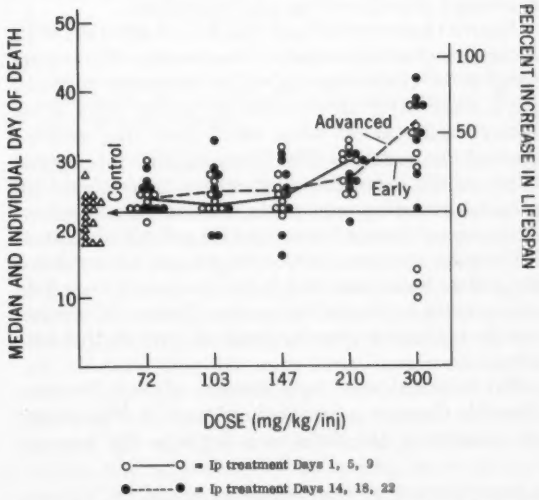

FIGURE 9.—Chemotherapeutic response of iv Lewis lung carcinoma to 5-FUdR.

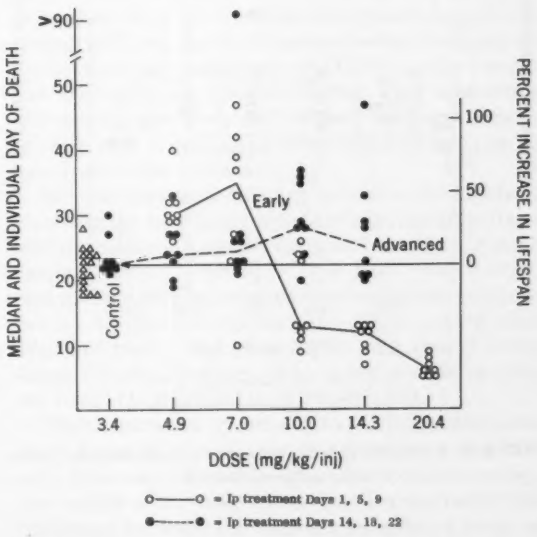

FIGURE 10.—Chemotherapeutic response of iv Lewis lung carcinoma to melphalan.

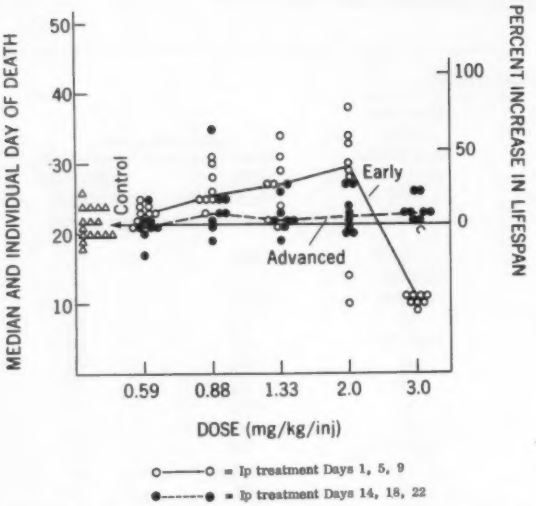

FIGURE 11.—Chemotherapeutic response of iv Lewis lung carcinoma to mechlorethamine.

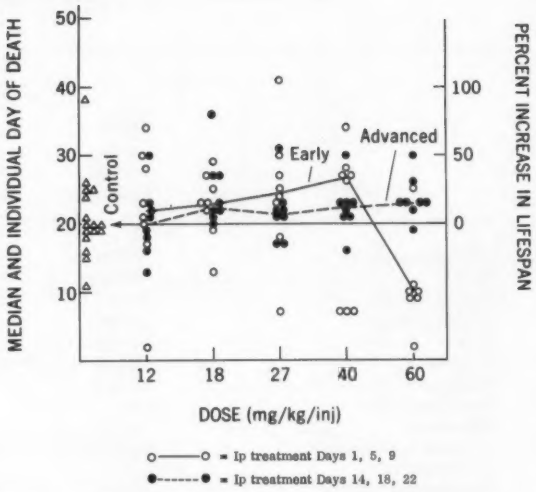

FIGURE 13.—Chemotherapeutic response of iv Lewis lung carcinoma to bleomycin.

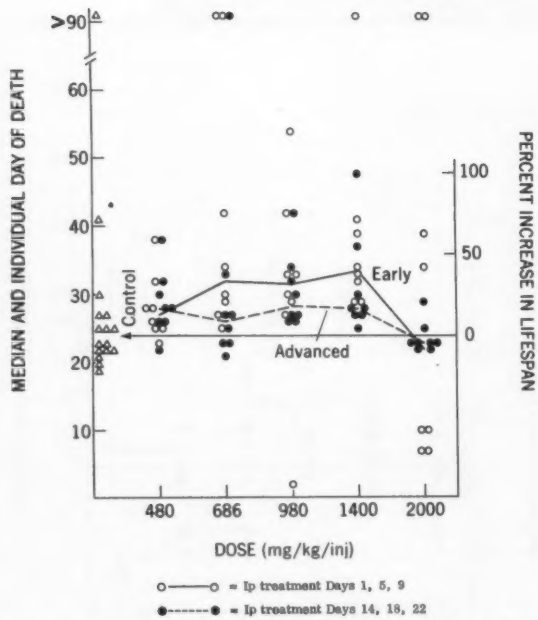

FIGURE 12.—Chemotherapeutic response of iv Lewis lung carcinoma to cytosine arabinoside.

Regression of Lung Tumors After Cyclophosphamide Treatment

Cyclophosphamide at doses of 133, 222, and 370 mg/kg was administered ip to mice bearing 14-day iv Lewis lung tumor and to normal (nontumored) mice to determine the effect of the drug on tumor growth in the lungs. Five mice from each group were killed and their lungs weighed at 2- or 3-day intervals starting 1 day after drug administration.

Figure 14 shows the lung weights on different days after cyclophosphamide treatment. The lung weights of tumor-bearing mice decreased for 7-10 days after drug administration (21-24 days after tumor implantation) after which time lung weights started to increase. The lung weights of normal mice, on the other hand, showed a dose-related increase continuing until 10 days after drug injection. The lungs of normal mice receiving the high dose of 370 mg/kg appeared hemorrhagic and edematous 5 days after treatment and, in many cases, serous fluid was present in the pleural cavity. This could account for the relatively heavier lungs of mice at this dose level.

The regression of lung tumors after cyclophosphamide therapy is depicted in figure 15. The weight of tumor was the difference between the average

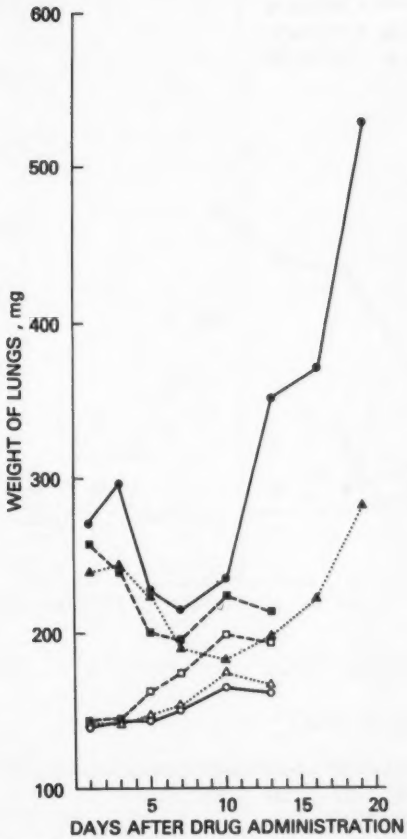

FIGURE 14.—Weight change in the lungs of tumor-bearing and nontumor-bearing mice after treatment with different doses of cyclophosphamide. Cyclophosphamide was administered on Day 14 after tumor implantation.

weights of the lungs of tumored mice and the lungs of normal mice receiving the same dose of drug. This was done because of the observed dose-related increase in the lung weights of normal mice treated with cyclophosphamide. There was a marked decrease in the tumor weights of mice treated with 222 mg/kg of cyclophosphamide from about 100 mg on the day of drug administration to a nadir of about 9 mg 10 days later (24 days after tumor transplantation). Relapse followed the initial period of inhibition and the tumor then started to resume growth. A dose of 133 mg/kg had relatively little effect on the tumor. Although the 370-mg/kg dose level did show marked inhibition of tumor growth, it also proved to be toxic as evidenced by the considerable body weight loss and high mortality rate of animals receiving the dose.

Combination Chemotherapy of Advanced Iv Lewis Lung Tumor With Cyclophosphamide and BCNU

Single drug treatment with either cyclophosphamide or BCNU was shown to be curative against early iv Lewis lung tumor and considerably increased lifespan on treatment of advanced tumor (figs 3 and 4). A combination treatment study with these two drugs was carried out against advanced tumor and the results are shown in figure 16. Treatment was ip on Day 14 only.

As before, either drug given alone was not curative. However, there was definite prolongation of lifespan on single drug therapy. Combined treatment with these two drugs brought about ILSs greater than either drug alone; some combinations gave one of eight 90-day survivors. The combination of 20 mg/kg of BCNU plus 200 mg/kg of cyclophosphamide was optimal with an ILS of 172%. This was about 60% greater than the maximal response to cyclophosphamide alone (108% ILS at 300 mg/kg).

DISCUSSION

Pulmonary growth of Lewis lung carcinoma develops when tumor cells are inoculated sc, im, or iv into the host mice. The tumor growth in the lungs after sc or im inoculation is a result of spontaneous metastasis from the sc or im primary tumor (3,7). On the other hand, the growth of lung tumor resulting from iv implantation is considered as induced or artificial metastasis and, in a strict sense, cannot be regarded as a model for metastasis (3,11,15). In the present investigation, we were concerned with evaluating this system as a model for primary lung cancer rather than as a metastasis model, with emphasis on its response to chemotherapy.

Most of the tumor cells implanted iv are deposited in the lungs as venous blood drains into this organ and the cells are arrested primarily in the lung capillaries (15,16). Tumor cells that have passed unarrested through the pulmonary circulation are transported by the bloodstream to other organs (18). Many of the iv implanted tumor cells perish in the lungs (15,16). However, only a few surviving cells are needed to produce tumor growth (16).

The progressive growth of Lewis lung carcinoma in the lungs of mice after iv implantation is quantifiable in terms of tumor weight. In the present study, the weight of the lung tumor was determined as the difference between the average weights of lungs of tumor-bearing mice and lungs of nontumored mice,

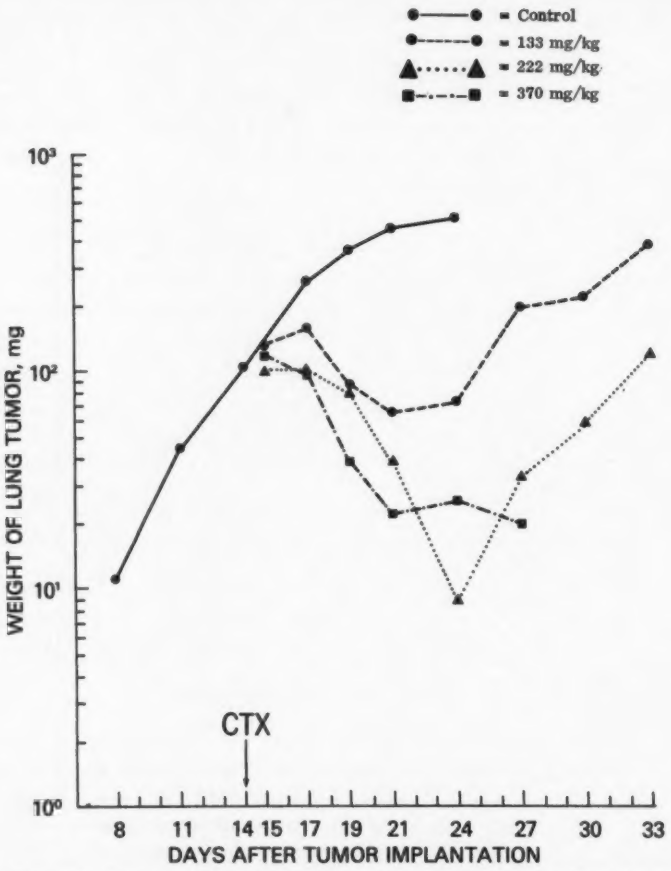

FIGURE 15.—Regression of lung tumors after treatment with different doses of cyclophosphamide.

assuming increases in weight due to lung tissue growth to be negligible (unpublished results in this laboratory show an average increase in normal lung weight of about 1.0 mg in a 2-week period). At the median day of death, the lung tumor weighed about 500 mg. Also, at this time the tumor colonies covered essentially the entire surface of the lungs.

The tumor burden in the lungs would appear to be the cause of death in mice bearing iv Lewis lung carcinoma since organ bioassay (table 2) demonstrated that the other organs, with the exception of kidneys, were usually not involved. Bonmassar et al (19), employing ^{125}IUdR uptake, concluded that intensive tumor growth occurred in the lungs 10-16 days after iv implantation of Lewis lung tumor cells and limited growth was found in the liver. Using a similar technique with B16 melanoma, Fidler (16) reported that by Day 14, no viable tumor cells could be found in organs other than the lungs. In the present study, kidneys harbored viable tumor cells although no visible tumor growth could be detected upon gross examination. This is in agreement with the observation of DeWys (20) that metastatic tumor cells were present in the kidneys of mice when assayed 17 days after im inoculation of Lewis lung carcinoma.

In general, Lewis lung tumor implanted iv is more responsive to chemotherapy than an sc or im implanted tumor. This is not unexpected since kinetic studies (12,13) demonstrated that pulmonary tumor

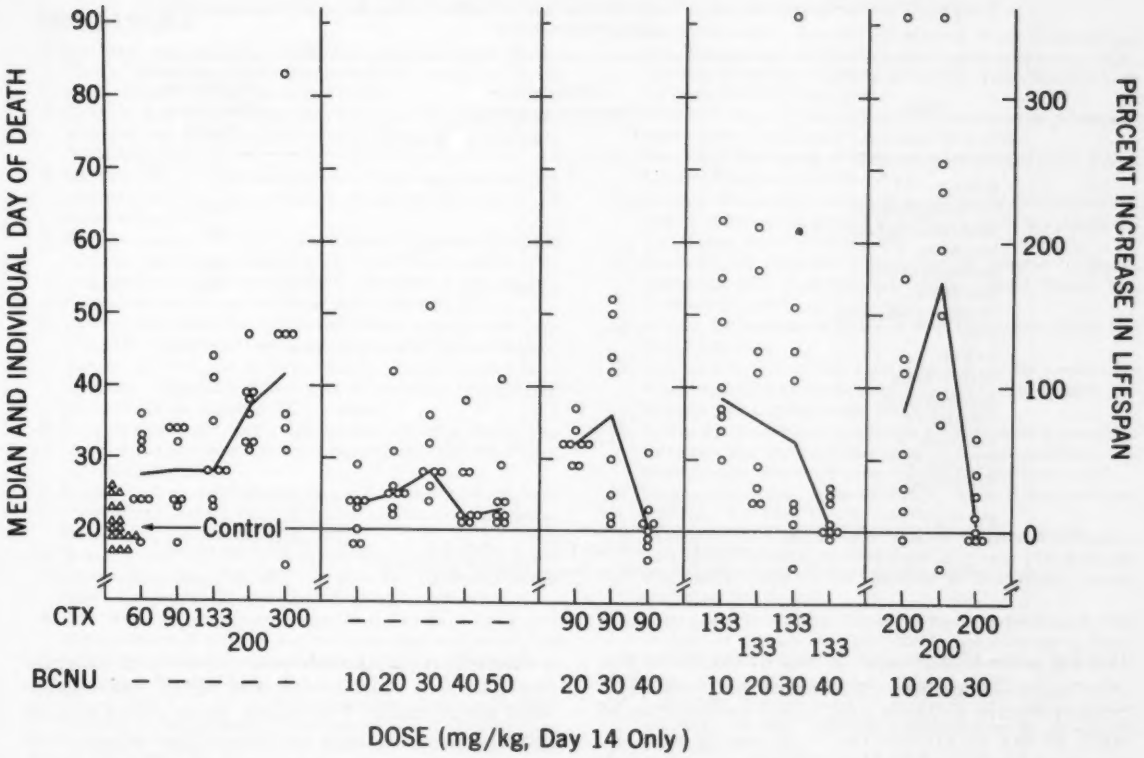

FIGURE 16.—Chemotherapeutic response of advanced iv Lewis lung carcinoma to combination treatment with cyclophosphamide and BCNU. Treatment ip on Day 14 only.

induced by iv implantation had a higher proliferative rate than primary sc (and presumably im) Lewis lung tumor. Furthermore, higher concentrations of drugs could be achieved in lung tumors as opposed to im or sc tumors due to the vascularity of lungs and the smaller mass of the individual lung tumor nodules. Table 5 summarizes the comparative response of iv, sc, and im Lewis lung tumor systems to the different drugs used in this study and also indicates activity of the drugs against human lung cancer. A notable finding in this study is the demonstration of activity for mechlorethamine against iv Lewis lung carcinoma. Mechlorethamine is one of the more active agents against human lung cancer and this drug has not yet been identified as active by either sc or im Lewis lung carcinoma. Another alkylating agent, melphalan, is also inactive against sc or im Lewis lung carcinoma but it has exhibited definite activity against iv Lewis lung carcinoma. In the clinic, melphalan is effective in the treatment of multiple myeloma and carcinoma of the breast and ovary but its activity against lung cancer has not been adequately evaluated (21). It is apparent from table 5 that, as a screen, iv Lewis lung tumor would have been more effective in identifying clinically active antitumor agents than either sc or im Lewis lung tumor.

It was noted for a number of drugs that some dose levels were toxic against early tumor but appeared to be active (ILS >25%) against advanced disease (figs 3, 4, 7, 9, and 10). For 5-FU (fig 7) for example, the dose of 67 mg/kg on Days 1,5, and 9 (early tumor) was toxic but the same dose showed significant activity in the treatment of advanced tumor on Days 14, 18, and 22. The MST of animals with advanced disease was 28.5 days (36% ILS) compared to 13.5 days for the early tumor group. Clearly, the longer MST of the advanced tumor group was a manifestation of late toxic deaths, treatment having begun on Day 14. The ILS compared to untreated controls indicates that there was antitumor activity but that the animals died primarily from drug toxicity.

Adriamycin was found to be highly active against early iv Lewis lung carcinoma and was significantly more effective when administered iv than ip (155%

TABLE 5.—Chemotherapeutic response of Lewis lung carcinoma at different inoculation sites compared with that of human cancer

Drug	Activity of Lewis lung tumor implanted—*			Palliative in human lung cancer
	Iv	Sc†	Im†	
BCNU	+++	++	++	
Cyclophosphamide	+++	++	++	Yes
Hexamethylmelamine	–	NT	–	Yes
Melphalan	++	–	–	
Mechlorethamine	+	–	–	Yes
ThioTEPA	–	NT	–	Yes
Cytosine arabinoside	+	+	–	
5-FU	++	–	–	
5-FUdR	++	+	+	
Methotrexate	–	–	–	Yes
Actinomycin D	–	–	–	
Adriamycin	+++	–	–	Yes
Bleomycin	+	++	++	
Vinblastine	–	–	–	
Vincristine	–	–	–	

*+++ = Curative on optimal schedule, ++ = >50% ILS, + = ≥25% ILS, – = <25% ILS, NT = not tested.
†Data of Mayo et al (3) and unpublished screening data of DR&DP, DCT, NCI.

ILS for iv vs 45% for ip). In another study in this laboratory (22) adriamycin administered iv on Day 1 to mice bearing iv Lewis lung tumor produced six of eight 90-day survivors. This is in accord with the report of Lenaz and Di Marco (23) that optimal effectiveness of adriamycin can be obtained when the iv treatment route is used against slow-growing solid tumors. These results are a further confirmation that route of administration is one of the factors that may determine the effectiveness of a drug.

Individually, cyclophosphamide and BCNU are curative against early iv Lewis lung tumor and show increased lifespan on treatment of advanced tumor. These two drugs were found to be therapeutically synergistic in combination against advanced tumor (fig 16). This indicates that iv Lewis lung carcinoma is adaptable to combination chemotherapy studies.

A single dose of cyclophosphamide against advanced tumor resulted in significant regression and effectively inhibited pulmonary tumor growth for a period of time (fig 15). It seems possible that subsequent treatment(s) with cyclophosphamide at about the nadir might elicit a prolonged period of tumor growth inhibition or even a cure. In administering cyclophosphamide to nontumored mice, we noted that a high dose (370 mg/kg) of the drug induced weight increases in the lungs of the mice (fig 14). These lung weight increases are a manifestation of cyclophosphamide toxicity in the form of pulmonary edema. Such toxic effects of cyclophosphamide have been observed in man (24) and animals (25-27).

Overall, iv implanted Lewis lung carcinoma appears to be a reproducible and highly quantifiable solid tumor model. This tumor model offers certain advantages over the sc and im systems presently in use in the screening program of the DR&DP, DCT, NCI. The utilization of the iv Lewis lung tumor as an experimental system for therapeutic investigations seems feasible and clinically relevant.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053
Adriamycin: NSC-123127; CAS reg. No. 23214-92-8
BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-
Bleomycin: NSC-125066
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate
Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; arabinosylcytosine
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8
5-FUdR: NSC-27640; uridine, 5-fluoro-2′-deoxy-
Hexamethylmelamine: NSC-13875; CAS reg. No. 645-05-6
Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2′-dichloro-*N*-methyl-, hydrochloride
Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[p-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-
Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[p-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-
ThioTEPA: NSC-6396; CAS reg. No. 52-24-4; phosphine sulfide, tris(1-aziridinyl)-
Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate
Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Biologic Characteristics of Tumor Progression in the Mouse Mammary Gland[1]

Daniel Medina[2,3]

SUMMARY

The murine mammary tumor system is characterized by the presence of a preneoplastic cell population, the hyperplastic alveolar nodule, which gives rise to mammary tumors. The biologic and tumor-producing characteristics of the preneoplastic nodule cell populations are reviewed with the idea that the system might serve as a model for the effects of therapeutic drugs on the growth of primary solid tumors and on the neoplastic transformation. Factors leading to the regression of preneoplastic cell populations might be a profitable avenue for future research.

[Cancer Chemother Rep Part 2, vol 5:127-131, 1975]

A consideration of the therapy of neoplasms, whether chemically, immunologically, or surgically mediated, has operated on the concept that the growing neoplasm is the appropriate and only target site. While this concept is understandable and, in most cases, appropriate, one should not lose sight of the fact that the process of neoplastic development is a multistage event. Upon exposure to an oncogen, a cell(s) undergoes a series of qualitative and quantitative changes which ultimately are expressed as a neoplasm. The concept that explains the qualitative changes that determine a neoplasm's behavior has been termed "tumor progression" by Foulds (1,2). Progression can occur at both the level of the established neoplasm and at any preneoplastic stage. The development of model systems to study the multistage process of the neoplastic transformation has been hampered by the inability to define, observe, and/or manipulate the intermediate cell populations. This has been particularly true for skin carcinogenesis where intermediate stages are postulated but are difficult to visualize.

The mammary gland and liver are the two major systems in which intermediate populations exist. The ability to transplant normal, preneoplastic, and neoplastic mammary tissues into their natural site, ie, the mammary fat pad, offers the investigator the advantage of studying the process of preneoplastic and neoplastic progression and its underlying mechanisms, as well as obtaining insight into the essential alterations of neoplasms. Alternatively, the same system offers several target sites for chemotherapeutic intervention. Thus, the preneoplastic cell population emerges as a potential target site for chemotherapy. In the following paragraphs, the biologic and tumor-producing properties of the preneoplastic mammary nodule cell populations are discussed.

MODEL SYSTEM

The scheme depicted in figure 1 summarizes the present state of our knowledge concerning the sequence of events in murine mammary tumorigenesis (3,4). The model demonstrates several concepts which are important in any discussion of mammary tumorigenesis. First, at least two sequential transformations occur in the process of mammary tumorigenesis. The first is the induction of nodule cells and has been termed the noduligenic transformation (3). This transformation leads to the appearance of subpopulations of mammary cells (nodules) which appear either as alveolar or ductal hyperplasias. The second transformation is the induction of neoplastic cells and has been termed the neoplastic transformation (3,4). Neoplastic cells generally arise from nodule cell populations although the model does not exclude one-step transformations from "normal" cell populations to neoplastic cells.

The second important concept that arises from this model concerns the action of oncogenic agents. Extensive data have demonstrated that both viral and chemical carcinogens can act at both the level of

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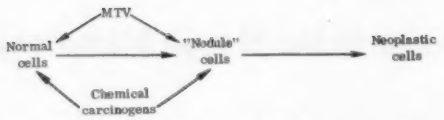

FIGURE 1.—Sequence of events in murine mammary tumorigenesis.

the normal cell population and the nodule cell population to induce and/or enhance subsequent noduligenesis and tumorigenesis, respectively (4,5). A corollary to the above concept would indicate that inhibitory agents could exert their effects at any stage along the progression sequence. Thus, it would be equally valid and important to consider the effects of agents on nodule inhibition and regression as on tumor inhibition and regression.

BIOLOGIC CHARACTERISTICS OF NODULES

The most frequently encountered nodule is termed the hyperplastic alveolar nodule (HAN). HAN resemble the normal mammary lobules of pregnant mice, however, they are identified as discrete foci of alveolar hyperplasias in the mammary glands of nonpregnant, nonlactating female mice. They are found in virtually all strains of mice, although their incidence and frequency vary depending on the status of the mammary tumor virus (MTV) infection (6), the breeding history (6,7), and the genetic background of the host (6-8). HAN can be induced in the absence of overt MTV infection by either chemical carcinogens such as 7,12-dimethylbenzanthracene (DMBA) (9,10), or by prolonged hormone stimulation (5,11). Regardless of the etiological agents involved in the induction of HAN, all HAN have similar growth and behavioral properties. The study of HAN is facilitated by the ability to transplant normal and nodule tissue into their normal anatomic site, ie, the mammary fat pad, in syngeneic mice. The anlage of the host's mammary duct in a 3-week-old mouse occupies approximately 15% of the mammary fat pad which is surgically removed, leaving the rest of the mammary fat pad to serve as a transplantation site (4,-12). Upon transplantation, HAN grow and fill the fat pad with alveolar epithelial hyperplasia. Once the entire fat pad is filled (approximately 10 weeks after transplantation), net growth ceases. At some time thereafter, months after transplantation, mammary tumors evolve as discrete foci from the nodule transplants.

The frequency of tumor formation is dependent upon the genotype of the nodule transplants (table 1). Nodules occurring in the MTV-positive C3H and Balb/cfC3H mice produce a high incidence of tumors upon transplantation, whereas nodules occurring in MTV-free C3Hf and C3H/2 mice and in DMBA-treated Lewis rats produce a low incidence of tumors (4).

GROWTH CHARACTERISTICS

Nodule outgrowth lines can be established through the serial transplantation of nodule tissue into the gland-free mammary fat pads of young syngeneic mice. The resultant lines can be considered as in vivo analogs of in vitro cell lines. This technique allows the establishment and comparison of nodule lines with low and high tumor-potentials induced by different oncogenic agents. Thus, in six MTV-free Balb/c nodule lines (two arising in retired breeders and four arising in carcinogen-treated mice) low and high tumor-potential lines were isolated (table 2). Nodule line D1 possesses a low tumor-potential, producing only 8% mammary tumors by 16 months, whereas nodule line C3 which produces 50% mammary tumors by 6 months represents a line with a high tumor-potential.

All HAN studied so far, regardless of their etiologic source, are capable of infinite propagation (13). These HAN can be serially transplanted in the fat pads of young syngeneic mice indefinitely, whereas the normal mammary gland generally dies out after

TABLE 1.—Tumor-producing capabilities of hyperplastic alveolar nodules

Strain	Transplant*	No. of tumors/ No. of transplants	Tumors produced (%)	Mean time of tumor appearance† (wks)
C3H	HAN	31/44	70	20.6
C3H	NMG	5/47	10	28.0
C3Hf	HAN	3/29	10	45.0
C3H/2	HAN	1/17	2	26.0
Balb/cfC3H	HAN	12/15	80	30.3
Lewis rat	HAN	8/37	22	ND
Lewis rat	NMG	0/37	0	ND

*HAN = hyperplastic alveolar nodule; NMG = normal mammary gland.
†ND = not determined.

TABLE 2.—Tumor-producing capabilities of spontaneously arising and chemical carcinogen-induced Balb/c mammary nodule outgrowth lines

Nodule line	Origin	No. of tumors/ No. of transplants	Tumors produced (%)	TE50* (wks)	Mean age of mice without tumors (wks)
D1	Spontaneous	5/62	8	—	71
D2	Spontaneous	45/89	51	40	43
C3	DMBA	81/104	78	27	33
C4	DMBA	38/54	70	30	37
C5	DMBA	19/22	86	26	40
C6	DMBA	4/17	24	—	35

*Time required for 50% of the transplants to produce mammary tumors.

five transplant generations (60 weeks). Lines D1 and D2 have been serially propagated in vivo for 12 and 10 years, respectively, and have retained their histologic characteristics and relative tumor-potentials since their inception.

Although HAN have acquired a limited autonomy from restraints on division potential (ie, they are capable of infinite propagation), they still retain a significant degree of responsiveness to local regulatory factors. This fact is readily apparent when considering the growth characteristics in vivo. HAN will only proliferate in mammary fat pads, not in subcutaneous white fat. The growth characteristics of HAN in other white fat depots have not been systematically investigated, therefore, it is unknown whether the mammary fat depot contains a specific set of factors regulating the growth of nodule cells. Secondly, HAN, but not mammary tumors arising from the nodules, are subject to growth inhibition by local growth regulators produced by mammary ducts growing in the mammary fat pads (4,14). Nodules transplanted into fat pads containing growing ducts fail to overgrow the ductal tissue and stop growing at the interface of the growing host ducts. The ability to override local growth regulatory factors remains an important distinction between nodules and neoplastic mammary tissue. The nature of the growth regulators operating in this system is currently unknown.

Most HAN arising in either MTV-positive C3H mice or MTV-free Balb/c mice, either "spontaneously" or carcinogen-induced, are independent of ovarian and adrenal hormones, but not of anterior pituitary hormones, for growth and tumorigenesis (4,15). Conversely, exogenously administered prolactin will enhance the tumorigenic transformation in ovariectomized-adrenalectomized mice (16). In accordance with this relative lack of sensitivity to ovarian hormones, most Balb/c HAN and mammary tumors have very low, barely detectable levels of cytoplasmic or nuclear localization of estradiol receptors (17). In this respect, murine mammary tumors differ markedly from chemical carcinogen-induced mammary tumors in the rat.

CELL POPULATION CHARACTERISTICS

A single primary nodule gives rise to a variety of outgrowths upon transplantation into the cleared fat pads of young, syngeneic mice. The variety of outgrowths with different growth characteristics produced by a single nodule are due to subpopulations of cells within the nodule that are characterized by a set of stable, inherent, independently assortable characteristics (4,18). Primary nodules can be subdivided to select for differences in growth rate, secretory status, tumor potential, and histogenetic appearance. The characteristics of individual nodule outgrowths are stable for each line and are retained indefinitely (4,18). These experiments suggest that a nodule is comprised of a heterogeneous population of cells. The characteristics of a given tumor will be determined by the properties of the group of cells in a nodule that progress to neoplasia.

The cellular components of nodule populations have not been extensively investigated. Slemmer (19) has proposed that HAN are comprised of three cell populations, ie, altered epithelial, normal epithelial, and myoepithelial. The epithelial cells can be divided into ductal and alveolar cells (19). In Slemmer's scheme, altered ductal cells give rise to ductal hyperplasias, altered alveolar cells give rise to hyperplastic alveolar nodules, and altered myoepithelial cells give rise to fibroadenomas and adenoacanthomas (19). Removal of the normal cell component from the nodule cell population resulted in nodule cell populations with markedly restricted growth. This observation suggests that normal cells exert a significant synergistic effect on the growth of nodule cells. Progression to adenocarcinomas was associated with loss of detectable normal epithelial cells. The role of myoepithelial cells is less defined. Slemmer postulated than HAN giving rise to squamous metaplasia

TABLE 3.—Effect of a single dose of cyclophosphamide on D2 tumor-potential

Group	Time interval after transplantation to cyclophosphamide administration (wks)	Fat pad filled (%)	No. of tumors/ No. of transplants	Tumors produced (%)	TE50* (wks)
1	3	30	8/15	53	41
2	8	80	17/21	81	34
3	13	100	16/22	73	28
4	Control	100	14/16	88	24

*Time required for 50% of the transplants to produce mammary tumors.

were comprised of myoepithelial cells (19). Electron microscopic investigations on the ultrastructure of cells giving rise to squamous metaplasia suggest that the epithelial glandular cells, not the myoepithelial cells, are the metaplastic cells.[4] The role of normal and myoepithelial cells in the progression from nodule to tumor phenotype needs further elaboration, if one wishes to treat these tissues therapeutically on a rational basis.

INHIBITION OF NODULE GROWTH

The inhibition of nodule growth and tumorigenesis has received little attention. Of the few studies, the experiments performed by Hui et al (20,21) deserve special comment. Both noduligenesis and tumorigenesis in MTV-positive C3H mice were inhibited by diets deficient in phenylalanine, an essential amino acid. The degree of inhibition was directly proportional to the dietary level of phenylalanine. The inhibition of tumors from transplanted nodules was directly proportional to the inhibition of nodule growth. The authors conclude that the inhibition was due to both restricted caloric intake and an altered hormonal milieu (20,21).

In a second study, nodule outgrowth lines in MTV-free Balb/c mice were treated with a single injection of cyclophosphamide[5] (20 mg/kg) at various intervals after transplantation (table 3). A single dose of cyclophosphamide significantly delayed the neoplastic transformation in nodule line D2, which has a relatively high tumor-potential. There was a positive correlation between the inhibition of nodule growth in the fat pad and the ultimate tumor incidence and latent period. Also, the delay in the latent period was directly proportional to the time of exposure to cyclophosphamide. The earlier the exposure time, the greater the delay in tumor appearance. These studies, one extensive and one very preliminary, indicate that tumorigenesis can be inhibited by various therapeutic manipulations. To what extent the transformation and growth of tumors can be markedly or permanently inhibited awaits further extensive experimentation.

DUCTAL HYPERPLASIAS

In addition to HAN, ductal hyperplasias are frequent in mice treated with chemical carcinogens (10). These lesions appear as atypical proliferations of mammary ducts with abundant intraluminal epithelial proliferations. Histologically, they appear as atypical ductal hyperplasias with varying degrees of atypia up to and including atypia characterized as ductal carcinoma in situ. Two ductal lines have been established as transplantable lines, but their preneoplastic nature and hormonal dependencies are still under preliminary investigation. Since they are predominantly seen in carcinogen-treated mice, are more abundant than HAN, and give rise to tumors similar to human breast ductal neoplasms, they may provide another model for use in therapeutic trials.

The data summarized in this paper suggest that the murine mammary tumor system may provide a system to investigate the effects of therapeutic drugs and manipulations on not only the growth of established tumors but on the transformation of the preneoplastic to the neoplastic phenotype. Efforts might be directed more profitably towards factors leading to the regression of nodule cell populations rather than the already established neoplastic cell population.

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[5]Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,-3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan.

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New Models for Cancer Chemotherapy[1]

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SUMMARY

Rodent tumor test systems have been utilized to identify the effectiveness of many of the present clinically administered chemotherapeutic agents. These experimental animal models have provided invaluable information towards predicting the degree of cytocidal action on given cell populations. Subsequent effects in the patient, however, have not always been comparable to those extrapolated from the laboratory. Our aim has been to devise alternative systems which may bear closer resemblance to the human situation with respect to morphology, tumor products, kinetics, and detoxification mechanisms. Some of the approaches presently adopted at the Chester Beatty Research Institute are discussed in this paper. These approaches depend on the ease with which various human tumors will grow and maintain their identity in immunologically subnormal animals. The purposes of the investigations are threefold and are interdependent: (*a*) the determination of what proportion of primary explants from certain tumors in man can be established in mice; (*b*) the development of transplantable xenografts from human tumors; and (*c*) the testing of both new and known chemotherapeutic agents for activity against these human tumors.

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MATERIALS AND METHODS

During the past 18 months, attempts have been made to gain an understanding of the xenografting potential of the various breast tumor types in different immunodeficient hosts. Breast tumors are difficult to grow outside their normal environment (1,2) and this is one of the reasons it was decided to concentrate predominantly on this tissue. Another reason is that breast carcinoma is one of the principal lines of interest of our Institute-Hospital complex. Table 1 shows the number of breast tumor specimens received with respect to the histologic grading of malignancy according to Bloom and Richardson (3), as well as the lymph node involvement, fibroblastic reaction, and lymphocytic infiltration.

The specimens consisted of primary infiltrating duct carcinoma of the breast and some metastatic breast tumor obtained surgically, the diagnosis always being confirmed histologically. Slices of the tumor were usually stored in medium 199 with added antibiotics at 4°C before collection. The tissue was implanted within 4-5 hours of removal from the patient. All surgical procedures in laboratory animals were executed under ether anesthesia and aseptic conditions. At postmortem examination, all graft remnants (whether they appeared to be growing or not) and any visibly abnormal organs were fixed in Bouin's fluid.

Initially, breast tumors were implanted into hamsters or mice that were immune deficient.

T-cell-deprived mice.—The type of immune-suppressed host that was used included T-cell-deprived mice (4-7). The immune-deprived mice used were prepared by thymectomizing 4-6-week-old CBA mice, administering 850 rads of whole-body irradiation 1 month later, and intravenously (iv) injecting syngeneic bone marrow. At least 1 week after irradiation, breast tumor pieces were implanted into from one to four sites depending on the amount of tissue available.

Deprived mice and colonic tumor.—An attempt was also made to induce immune paralysis to human antigens in these mice. A piece of breast tumor was implanted on the side opposite to a human colonic tumor of 3-4-week growth in the same animal. We felt that any antihuman antibody that formed might be absorbed by the established colonic tumor, which would leave the breast graft under weaker attack.

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TABLE 1.—Histologic description of breast tumor specimens received

Malignancy grading*	Lymph node involvement	Incidence of graded specimens with—					
		Lymphocytic infiltration			Fibrous stroma		
		Dense	Moderate	Minimal	Dense	Moderate	Minimal
I (3 specimens)	0	0	1	2	2	1	0
II (38 specimens)	30	2	10	26	23	10	5
III (4 specimens)	3	0	2	2	1	2	1

* Histologic grading of malignancy according to Bloom and Richardson (ref 3).

Deprived mice and sham excision.—The tumor was implanted under a zone of granulation tissue produced in the inguinal region of deprived mice 5 days after bilateral 2 × 10-mm full-thickness portions of skin were excised. It was thought that the increased vascularity at this site and the subsequent healing of the wound with setting down of fibrous stroma might provide a better environment for growth.

Kidney capsule site in deprived mice.—The right kidney capsules of deprived mice were also used as a site for implantation of 1-mm^3 fragments of breast tissue. Double and Ball[5] have grown colonic carcinoma consistently at this site, but histologically our remnants did not show any tumor.

Normal neonates.—CBA neonates were injected with 0.05 ml of tumor-cell suspension which had been prepared from cells spilled off while slicing the tissue. Again, no growth had occurred at 3 months as revealed at postmortem examination.

Intracerebral implantation in normal CBA mice.—Intracerebral implantation of 1-mm^3 fragments of breast tumor into normal CBA mice was attempted. The graft was placed under the right dorsal parietal plate region after removing a piece of bone from the skull. This method was tried because Chesterman (8) successfully grew human oat cell bronchial carcinoma at this site which is thought to be immunologically privileged because of the blood-brain barrier.

Hamsters.—Irradiated and nonirradiated cortisonized hamsters were also used. Adult Syrian cream hamsters were divided into two groups: those receiving 3 mg/kg of body weight of cortisone acetate intraperitoneally (ip) at the time of implantation and twice weekly thereafter, and those receiving 400 rads of whole-body irradiation 2-3 days prior to implantation plus 3 mg/kg twice per week after implantation. Sites for implantation were subcutaneous (sc) and cheek pouch. Again, we had no marked improvement in our results although many fibrous remnants were identified at postmortem examination. It should be emphasized, however, that relatively few trials of these modified methods were made and the fact that breast tumors are not predisposed to xenografting must be considered when assessing the usefulness of the methods. Negligible takes resulted in all of these early studies in which nearly 100 breast tumors were implanted. Subsequently, it was decided to assess the congenitally athymic nude mouse as a recipient, particularly for breast carcinoma, since other researchers have demonstrated human tumor growth in such animals (9,10).

Nude mice.—In our effort to standardize transplantation conditions, blocks of tissue from a single tumor were implanted sc into two axillary and two inguinal regions of four female nude mice, which usually provided 16 growth trials for every tumor received. The nude mice used had been randomly bred by various accredited breeders and were delivered to our laboratory as specific pathogen free. According to the experience of other researchers, nude mice live only 3-5 months in conventional animal house conditions and are very susceptible to viral, bacterial, and parasitic infestations, presumably because of their impaired immunity (11,12). Our experiments required a minimum lifespan of 9 months to enable us to perform a postmortem examination during any period up to 6 months after implantation of the breast sample. Assuming that these mice could harbor progressively growing breast carcinoma, 6 months seemed a reasonable time period for grafts to reach transplantable size. There are several ways

[5]Double JA, and Ball CR. Influence of transplantation site on the establishment of human tumour xenografts in immunosuppressed mice. Presented at the 1974 Autumn meeting of the British Association of Cancer Research Workshop.

of extending the longevity of nude mice and we chose filter boxes since these were the most effective method of maintenance in the conventional animal room available to us. The day-to-day cleaning and the implantations were performed inside plastic isolators; everything coming into direct contact with these mice was sterilized.

RESULTS AND DISCUSSION

The results are summarized in table 2. The percentage of graft "takes" was calculated from the total number of grafts that were tumor-positive at postmortem examination divided by the total number of grafts implanted in the rodents. Comparison of these results with those of xenografting into other kinds of immune-deficient mice shows that nude mice provide the most favorable environment for tumor growth.

The criteria which we feel indicate successful xenografting of human tumor to experimental animals are as follows: (*a*) the patient's surgically removed specimen and the implanted specimen at postmortem examination of the animal should have similar morphology as judged by histopathologic examination; (*b*) progressive growth should occur, usually characterized by an increase in size and the presence of mitotic activity in the grafted tumor; (*c*) the implanted tumor can be passaged; (*d*) ideally, the metastasis of the grafted tumor will result in the death of the host; and (*e*) if possible, the demonstration of human chromosomes in metaphase spreads in the implanted tumor.

So far, the breast carcinoma xenografts have not satisfied all of these criteria. A breast cell line, HBT3 (13) (initially grown in vitro), and a transplantable bronchial xenograft have metastasized to nude mouse lung tissue and T-cell-deprived mouse lung tissue, respectively.

Further details of the breast xenografts in nude mice are given in table 3 and serve to illustrate the extent to which the breast xenografts have fulfilled the criteria outlined above. The incidence of "takes" at 3- and 6-month periods were similar for the same tumor. The percent of remnants was 66% at 3 months and 61% at 6 months for the same 48 breast tumors considered in table 3. The remnants cover three categories of result:

(*A*) The takes under the heading "tumor-positive takes" in table 3 are covered by our first and second criteria for successful xenografting, ie, there is a similar morphology between the patient's tumor and the xenograft with the presence of hyperchromatic nuclei and dividing cells in the graft in the mouse although it may not have appreciably increased in size at the time of postmortem examination. This suggests that cell production is equivalent to cell loss or that the cells are in a quiescent or regressing phase. The percent of takes is the same at 3 and 6 months as revealed at postmortem examination for the same tumor in this group.

TABLE 2.—Summary of implantation results

Type of host	No. of specimen takes/ total No. of specimens implanted	Specimen takes (%)	No. of graft takes/ total No. of grafts implanted	Graft takes (%)
Deprived mice	2/56	3.5	3/190	2
Deprived mice + colonic tumor	0/10	0	0/40	0
Deprived mice + sham excision	0/8	0	0/54	0
Kidney capsule of deprived mice	0/5	0	0/12	0
Neonatal litters	0/5	0	0/40	0
Hamsters	1/22	4.5	1/170	0.6
Intracerebral implantation	0/8	0	0/24	0
Nude mice	27/48	56	179/640	28

TABLE 3.—Xenografts of 48 breast tumors in nude mice

Time in mos at postmortem examination	Remnants (%)	Tumor-positive takes (%)	Transplantable takes (%)	Total tumor takes (%)
3	66	15	10	25
6	61	15	16	31

(*B*) The "transplantable takes" are covered by the first, second, and third criteria for successful xenografting. They not only are morphologically similar to the patient's tumor with regard to mitotic activity but they have very definitely increased in size. They are at least 7-8 mm in diameter at 6 months, and in most cases have been passaged further. At postmortem examination, the percent of transplantable grafts is 10% at 3 months and 16% at 6 months. This is the type of "take" we are aiming for. An ability to passage the tumor is essential, since it is the only means of accumulating enough material to do further work. The transplantable takes have tended to result from the intraduct component of duct carcinoma, the more cellular and mucoid tumors. A tentative correlation seems to exist between a high degree of malignancy in the patient and the likelihood of the graft becoming transplantable in the mouse.

(*C*) The remaining remnants were not histologically tumor-positive but were mainly composed of fibrous tissue.

Some examples of histology of the patient's specimen and the xenograft are compared in figures 1-3. Morphologic examination of the takes shows a similarity between the specimen from the patient and the tumor grown in the nude mouse, although the grafts in the mice have tended to contain many more aberrant mitotic figures. The "comedo" carcinoma pattern, when present in the patient, grew in the mouse more readily than the infiltrating parts of the tumor. There are several inherent variables in our system. The variation in cellularity within a breast specimen from the same patient is unavoidable. A second variable is the fact we are using randomly bred mice.

In the future, two objectives could be pursued. First, the effects of different concentrations of hormones such as prolactin and 17β-estradiol should be tested on growing and resting grafts, and on normal and benign tissue. Second, breast cell lines must be developed for chemotherapy testing. In this context, Povlsen et al (14) have already exploited the human tumor in the nude mouse system, to some extent, and have shown a pattern of drug susceptibility of the xenografts in accordance with clinical practice.

Similarly, the testing of a number of known antitumor agents in a series of human tumors xenografted to T-lymphocyte-deprived mice has begun at the Chester Beatty Research Institute and the results are summarized in table 4. From this table the re-

TABLE 4.—Results of known anticancer agents against human tumors xenografted to T-cell-deprived mice*

Drug	Human tumor xenografts of—†					
	Colon 116	Colon 76	Bronchus 246	Bronchus (Mount)	Ovary 210	Kidney 381
Methotrexate	±	NR		±		
Cyclophosphamide			NR	NR	+	NR
Melphalan					+	
Sulfadiazenetriazene	±		±		±	
BCNU	+	+				
Cis-dichlorodiammineplatinum (II)					NR	
Adriamycin	±			NR		
Hexamethylmelamine	±		++	+	±	++
5-FU	+	NR		NR		

*Mitchley BCV, Clarke SA, and Connors TA. Unpublished data.
†Definitions: NR = no response; ± = slight response; + = tumor inhibition; ++ = tumor regression.

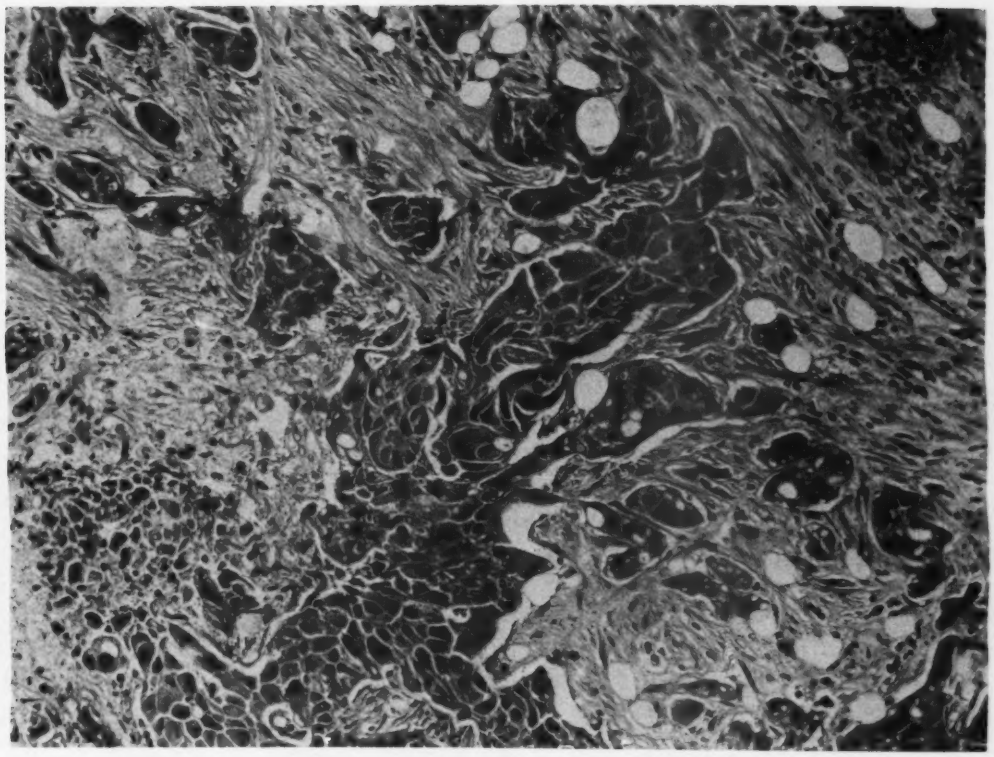

FIGURE 1A.—S37 patient. Infiltrating duct carcinoma of the breast (grade III) showing the infiltrating part of the tumor. There were also "comedo" carcinoma regions in other parts of the specimen removed from the patient at time of surgery.

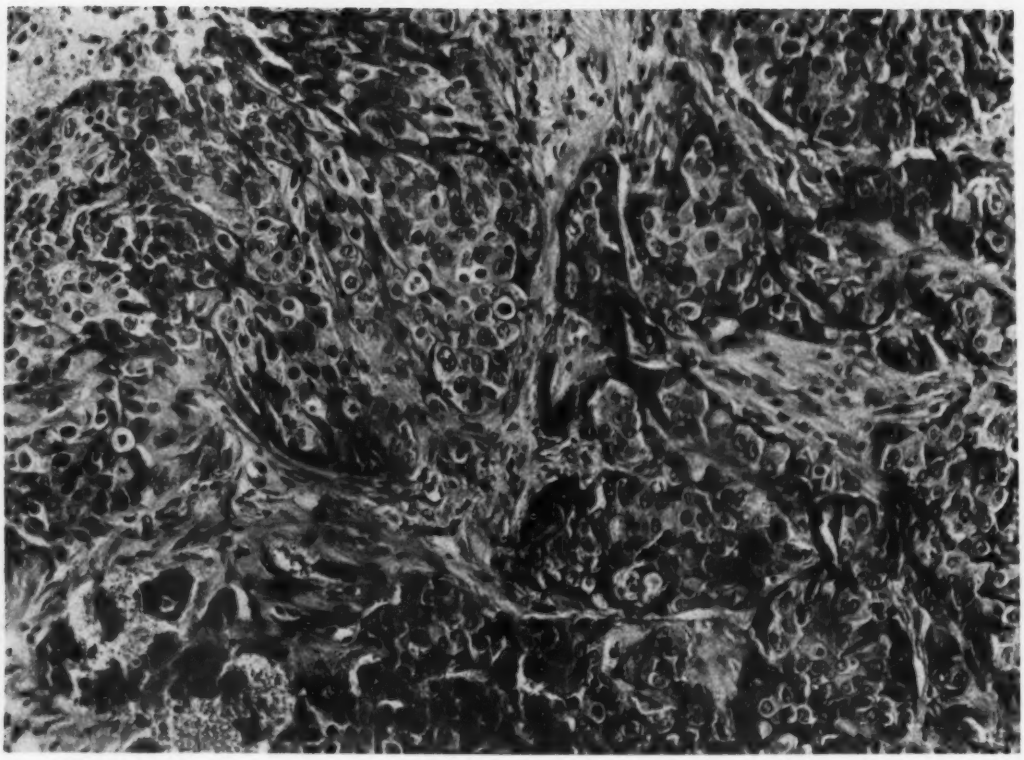

FIGURE 1B.—S37 nude mouse. Transplantable xenograft showing similar histologic pattern. This is from the same tumor as shown in fig 1A 6 months after implantation to nude mouse. "Comedo" carcinoma pattern was seen in other parts of the tumor.

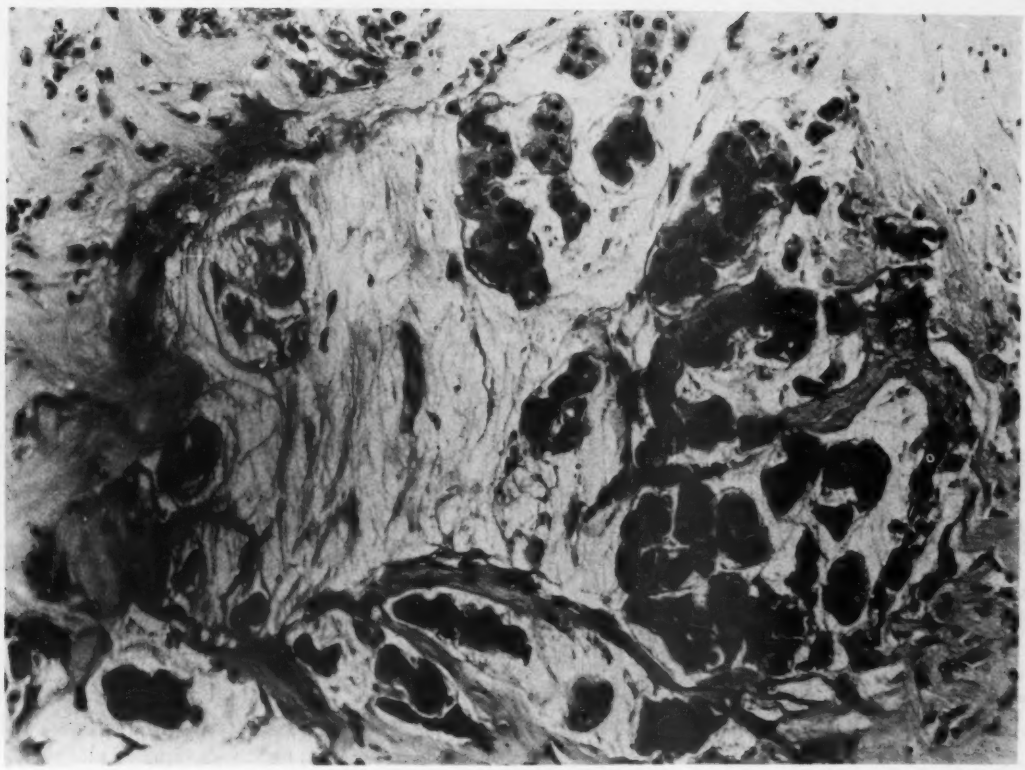

FIGURE 2A.—S47 patient. Mucoid infiltrating duct carcinoma of the breast (grade II) removed from the patient at time of surgery.

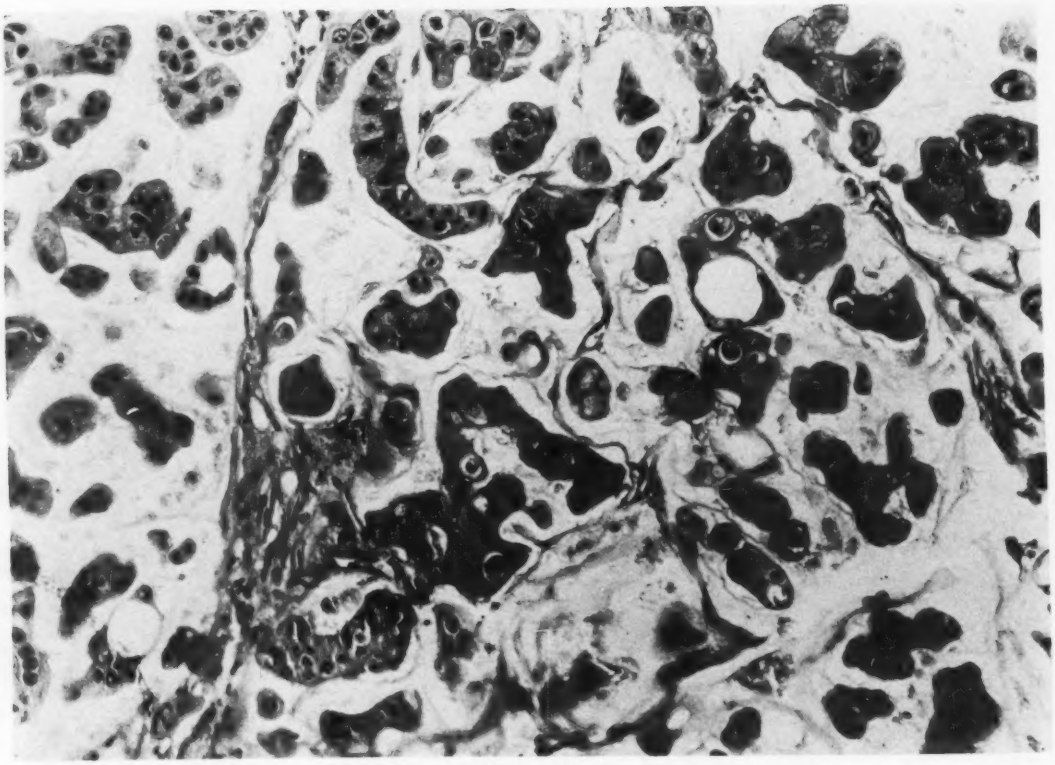

FIGURE 2B.—S47 nude mouse. Transplantable xenograft showing similar histologic pattern but growing in the nude mouse 6 months after implantation. This is from the same tumor as shown in fig 2A.

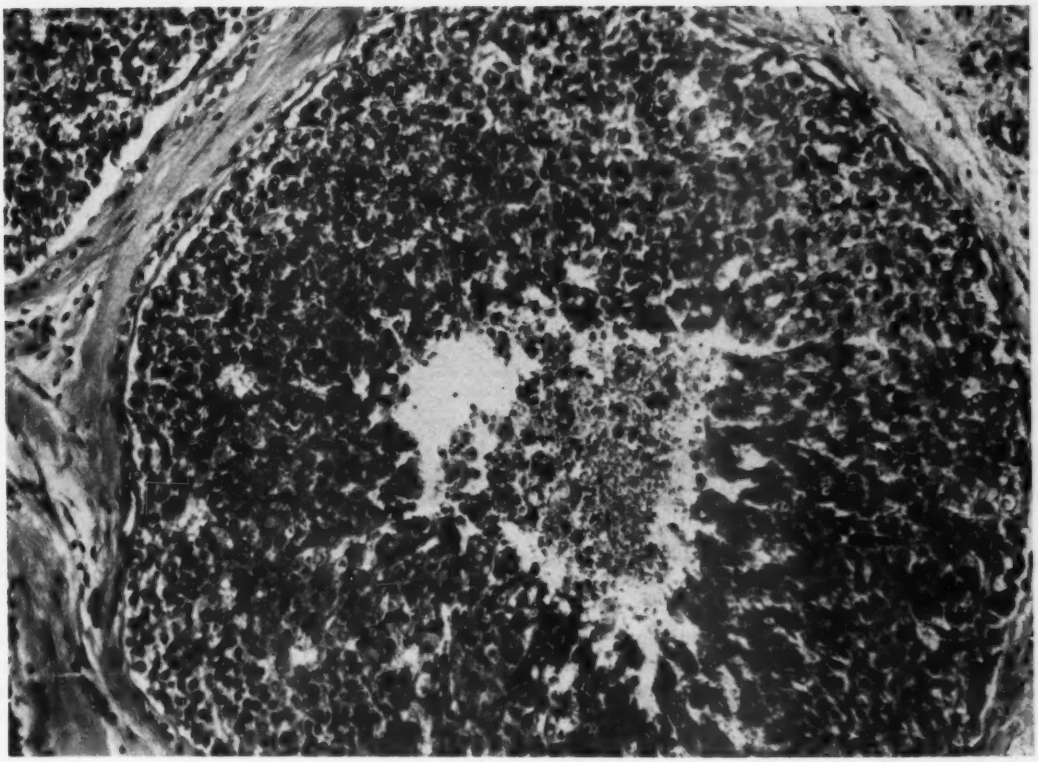

FIGURE 3A.—S62 patient. Infiltrating duct carcinoma of the breast (grade II) with large intraduct component showing "comedo" pattern in the specimen removed from the patient.

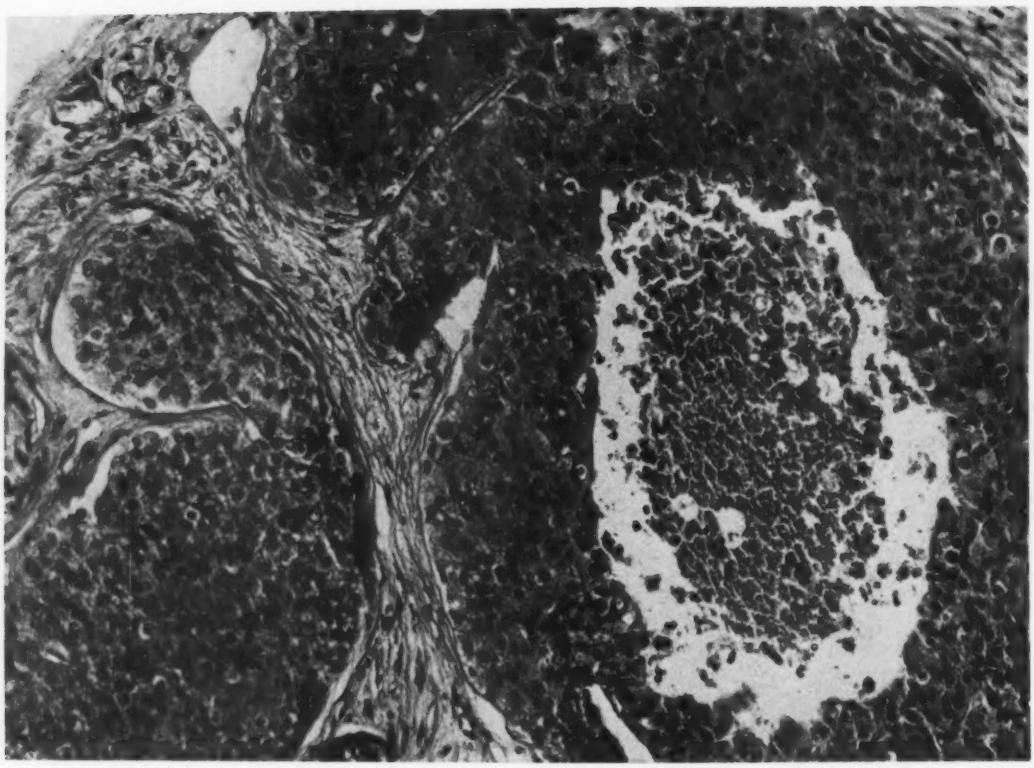

FIGURE 3B.—S62 nude mouse. Xenograft showing very similar histologic pattern after 6-months' growth in the nude mouse. This is from the same tumor as shown in fig 3A.

sponse of the ovarian tumor to the nitrogen mustards and the platinum compound is equivocal. The bronchial xenografts were fairly resistant to agents tested so far, including cyclophosphamide and adriamycin. However, both tumors responded well to hexamethylmelamine. This result was of interest since this compound had little or no activity against most experimental animal tumors yet is definitely active against lung cancer in man (15). A further point of interest was the broad spectrum of action of hexamethylmelamine which also inhibited the growth of a transplanted ovarian tumor and caused regression of a hypernephroma.

Kopper and Steele (16) have also obtained therapeutic responses in tumors between their fourth and 14th passage growing in deprived mice. In general, the response of the colonic xenografts to 5-fluorouracil (5-FU) was analogous to that seen by the cancer chemotherapist in the clinic.

The ability of nude mice and T-cell-deprived mice to withstand effective doses of chemotherapeutic agents has been previously described (14,17,18). We feel the human tumor growing in the T-cell-deprived mouse or nude mouse system has potential as a new model for secondary screening of selected anticancer agents. In particular, our results indicate that the nude mouse is a more favorable host for some breast carcinomas.

In conclusion, further definition of these model systems is required.

LIST OF COMPOUNDS

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

***Cis*-dichlorodiammineplatinum(II):** NSC-119875; CAS reg. No. 15663-27-1

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

Hexamethylmelamine: NSC-13875; CAS reg. No. 645-05-6

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

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Preliminary Drug Trials in a Renal Cell Carcinoma Animal Model[1,2]

Alan M. Shefner[3,4] and Marilyn Marlow[3]

SUMMARY

A transplantable renal cell carcinoma of the BALB/c mouse has been used for the evaluation of seven chemotherapeutic agents. This model appears to resemble the clinical situation of disseminated renal cell carcinoma in man. Although drug trials are still in progress, preliminary indications of the activity or inactivity of the drugs tested, at the doses and regimens used, are available. Bleomycin, hydroxyurea, and cyclophosphamide had little effect on survival time or on tumor growth. Adriamycin and vinblastine increased survival time by >25% and adriamycin appears to produce some reduction in tumor size. The two nitrosoureas, methyl-CCNU and BCNU, are highly effective in reducing tumor size and have produced a >25% increase in life-span to date.

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Human renal cell carcinoma, if inoperable or with widespread metastases, yields very few long-term survivors. Five-year survival rates of <5% have been reported in cases of metastatic disease (1,2). The disease is unpredictable; it may progress, remit, and, in very rare cases, undergo spontaneous regression (3,4). Spontaneous regressions of pulmonary and bone lesions after radical nephrectomy have been reported, possibly indicating a hormonal or immunologic control mechanism (5). Removal of the primary renal tumor in the absence of metastasis has resulted in 3-year survival rates of 45% and 5-year survival rates of 34%. These long-term survival rates were not affected by chemotherapy (5). Generally, standard chemotherapy has not been effective against metastatic renal cell carcinoma (6). Carter has reported on the nearly total failure of chemotherapy against renal cell carcinoma; only five standard agents have been evaluated and none were active.[5] Hormonal therapy has been reported to produce both favorable as well as poor results (7,8).

Few animal models for renal cell carcinoma have been described. In the past year we have been studying a murine renal cell carcinoma model system which was developed and characterized by Murphy and Hrushesky (9). The host is the BALB/c mouse. The tumor originated as a spontaneous renal cortical adenocarcinoma of the granular cell type (9). It develops after intrarenal (ir), im, ip, iv, and sc transplantation. It is quite slow-growing, with the survival time depending upon the site of inoculation. The purpose of our study is to characterize the survival response of the animals after various routes of inoculation (particularly ir and sc), to study the growth patterns of the tumor, and to determine the response of this tumor to selected chemotherapeutic agents leading to the possible utilization of this system for specialized drug testing.

METHODS

Male and female BALB/c mice, at least 6 weeks old, have been used with no differences noted in tumor growth or survival time.

For ir transplant a tumor cell suspension is prepared. Our procedure for preparing and inoculating the renal tumor is essentially that described by Murphy and Hrushesky with the exception that we transplant the tumor under the capsule of only one kidney (9). After the donor animal is killed, the tumor mass is aseptically removed and separated from the remaining kidney tissue. The tumor is weighed and, using a glass tissue grinder, a 1:10 homogenate is prepared with McCoy's medium containing 5% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The tumor preparation

[1]Supported by contract N01-CM-81021 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2]The tumor was generously supplied to us, in intrarenally transplanted donor mice, by Dr. Gerald P. Murphy, Roswell Park Memorial Institute, Buffalo, NY.

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[5]Carter SK. Report of the Associate Director. *In* Report of the Division of Cancer Treatment, NCI, 1974. Bethesda, Md, NCI, 1974. vol 1, pp 6.1-6.11.

and all materials are kept well chilled. Cell counts are routinely performed. Mice which are to be inoculated ir are anesthetized, a small incision is made, and the left kidney is exposed. A volume of 0.1 ml of homogenate is inoculated under the capsule of the kidney using a 27-gauge needle. The incision is closed with autoclips. After sufficient experience, the surgical procedure requires approximately 2-3 minutes per mouse and the mice recover within 10-15 minutes.

An sc implant of the renal cell carcinoma has been made by trocared fragment or sc inoculation with the tumor cell homogenate as prepared above.

CHARACTERISTICS OF THE MODEL

Murphy and Hrushesky (9) have characterized the murine renal cell carcinoma model in terms of the kinetics of tumor growth and metastasis using different transplantation routes and in terms of survival of transplanted mice. The tumor arose spontaneously as a renal cortical adenocarcinoma and was passaged sc approximately every 35 days. The tumor grows equally well when passaged into male or female BALB/c mice. Swiss mice challenged with as many as 1×10^6 tumor cells show no evidence of tumor growth. The metastatic index and tumor weights of recipient BALB/c mice inoculated ir with tumor suspensions ranging from 10^5 to as low as 50 viable cells correspond directly to the number of cells inoculated. Small localized tumors were found in 100% of the mice transplanted with 50 cells. Large tumors developed in 100% of recipient mice transplanted with 10^5 cells when the injection route was ir, ip, iv, im, or sc. The metastatic index was high only after ir transplant. Ir transplanted mice survived for an average of 46 days, while the median survival times were >60-75 days when other injection routes were used.

By Day 21 after ir transplant, all animals had metastatic tumor and 50% were grossly palpable. Tumor weight doubling time was approximately 7 days during the period of 21-35 days after inoculation.

Renal tumor growth was enhanced by the administration of testosterone or diethylstilbestrol but was unaffected by medroxyprogesterone, a progestational agent. Cell-free extracts of renal tumor did not produce tumor growth in 20-hour-old BALB/c mice who were ip inoculated or in 6-week-old BALB/c males or Swiss mice who were ir inoculated.

In our laboratory we have carried the tumor through 13 generations by ir transplant. The median survival times (MST) for generations three to 13 average 45.3 days and are in good agreement with the findings of Murphy and Hrushesky (9). All recipient mice from the fourth generation onward have developed tumor. We have also carried the tumor by sc transplantation of trocared fragments through ten generations; the MSTs for generations three to ten average 64 days. These MSTs agree well with our experience with sc transplant with tumor cell suspensions in which the MSTs for three experiments were 77.0, 54.0, and 49.8 days.

We have attempted to delineate the growth of the sc implanted renal tumor by calculating tumor weights from periodic caliper measurements of both homogenate and fragment-induced tumors. Tumor weights were calculated using the formula:

$$\frac{\text{length(mm)} \times (\text{width[mm]})^2}{2}$$

In the period of 21-42 days after implant, the time for a doubling of tumor mass is approximately 7 days.

The calculated weights for the two sc tumor groups are generally in the same range, with the homogenate-inoculated mice having somewhat larger tumors (tables 1 and 2). For both groups, there is a wide spread in tumor size at each interval. In the group of 60 homogenate-inoculated mice, one mouse had a tumor which was palpable but not measurable through Day 56. We had hoped that the size of the sc tumors would be more consistent when a homogeneous cell-suspension inoculum was used, but this has not yet been demonstrated.

In addition to the wide variability in size, another major problem with both of the sc implants is the tumor ulceration which often occurs as early as 3 weeks when the tumors are still quite small and develops in essentially all of the mice before death. This makes progressive tumor measurement difficult

TABLE 1.—Calculated tumor weights of sc inoculated renal tumor homogenate (60 mice)

Day after implant	Weight (g)		
	Range of mean tumor weights of cage groups*	Overall range	Average
23	>0.039-0.289	>0.0-0.527	>0.172
28	>0.087-0.484	>0.0-0.827	>0.301
34	0.174-0.899	>0.0-1.521	>0.536
40	0.304-1.495	>0.0-2.458	>0.808
49	0.379-3.805	>0.0-5.054	>1.533
53	0.398-4.034	>0.0-5.596	>1.828
56	0.510-3.837	>0.0-6.700	>2.187

*5 mice per cage group.

TABLE 2.—Calculated tumor weights from renal tumor SC03 (trocar)

Day after implant	Weight (g)	
	Range	Average
12	>0-0.056	0.011
14	>0-0.077	0.031
18	>0-0.137	0.076
21	>0-0.301	0.138
25	>0-0.493	0.196
29	0.046-0.564	0.260
33	0.060-0.938	0.440
39	0.169-2.160	0.900
42	0.267-3.092	1.090
48	0.329-2.246*	1.168
54	0.350-2.769	1.500

*Mouse with 3.092-g tumor died on Day 48.

or impossible and introduces the factor of bacterial infection.

We have also inoculated one group of mice ip with 0.5 ml of the 1:10 renal tumor homogenate which yields an MST of 34.7 days and 100% deaths by Day 58. This was approximately a five times larger cell inoculum than that used by Murphy and Hrushesky who reported >50% survivors at 60 days with ip inoculation of 10^5 tumor cells (9).

CHEMOTHERAPEUTIC TRIALS

Chemotherapeutic trials in this model have been quite limited. Based on clinical reports of activity against metastatic renal cell carcinoma, Hrushesky and Murphy tested CCNU and vinblastine against murine renal cell carcinoma (10). CCNU had yielded objective remissions in 20% of the patients in a study by Mittelman et al (11), and vinblastine had produced an approximately 30% rate of objective response (5). Hrushesky and Murphy evaluated the response to drug therapy by three criteria: tumor weight, metastasis, and survival time. Both drugs were given ip every seventh day (q7d) starting on Day 7 after tumor implant; in some experiments the drugs were given q7d starting on Day 21 when the renal tumor is palpable and metastasis has begun.

Tumor weight, as determined by autopsy at 40 days, was significantly reduced by both drugs when treatment was started on Day 7. The effect was dose related. A 75% reduction in tumor size with CCNU and a 95% reduction in tumor size with vinblastine were the best responses as compared to controls.

Lung metastases were determined microscopically after autopsy at 40 days. Both drugs were given q7d starting on Day 7. With CCNU given at 1.0 mg of drug/animal/injection (40 mg/kg), 80% of the animals had distant metastases at Day 40, and with vinblastine given at 0.02 mg of drug/animal/injection (0.8 mg/kg), only 50% had pulmonary metastases. All of the control mice had metastases.

In their survival time study, both drugs were given q7d starting on Day 7 or on Day 21. With CCNU, all three doses (0.4, 1.0, and 2.0 mg/kg) on both schedules increased survival time, with delayed therapy yielding better responses than earlier therapy. The optimum response was a 61% increase in mean survival time (47 days for controls and 76 days for CCNU-treated mice). With vinblastine, all doses (0.2, 0.4, and 0.8 mg/kg) and both schedules increased survival time, with the early treatment generally better than the delayed treatment. The optimum response was a >100% increase in mean survival time (45 days for the controls and 92 days for the vinblastine-treated group).

We currently have seven drugs on test in the renal cell carcinoma system in ir tumored mice. Bleomycin, adriamycin, and hydroxyurea were given on a daily schedule on Days 7-15. Vinblastine, cyclophosphamide, methyl-CCNU, and BCNU were administered q7d starting on Day 7 for five doses. All were tested at three dose levels. Each test group consisted of ten mice and a control group of 30 mice was used. Although the drug trials are still in progress (Day 50, control MST = 40.0 days), the data in table 3 allow certain conclusions to be drawn.

Bleomycin, hydroxyurea, and cyclophosphamide were ineffective in producing increases in survival times at the doses and regimens used in this experiment. Cyclophosphamide did appear to exert an effect on tumor growth during the period of drug treatment. Adriamycin showed some therapeutic effects at all dose levels that were tested. At the highest dose tested, 1.0 mg/kg/day, all ten mice were still alive at Day 50 and nine appeared to be bearing somewhat smaller tumors by palpation.

Vinblastine was not as effective in our drug trial as it was in the report of Hrushesky and Murphy (10) although the same dosages and treatment schedules were used. The two lower doses (0.2 and 0.4 mg/kg) did not increase the MST appreciably (10% and 7%). At the highest dose, 0.8 mg/kg, nine mice still survive but appear to be bearing large tumors. In the study of Hrushesky and Murphy (10), tumor weights were determined in animals killed at Day 40, 5 days after the last drug dose. Our comments on tumor size refer to palpable masses some 15 days after the last drug treatment. Thus the difference in the effect on tumor weight may be an indication of escape of residual tumor from drug control but the differences in survival time found at lower doses cannot be similarly explained.

As of Day 50, the two nitrosoureas, methyl-CCNU and BCNU, were the most effective drugs used in our

TABLE 3.—Interim results of first chemotherapeutic trial on Day 50 of test*

Drug and regimen	Dose range (mg/kg)	Comments
Bleomycin, qd Days 7-15	6.0-1.5	All doses inactive, T/C <125%; tumor growth generally parallel to that of controls
Adriamycin, qd Days 7-15	1.0-0.25	All doses, T/C≥125%; tumors generally smaller than in controls, especially at highest dose; 10 survivors at highest dose
Hydroxyurea qd Days 7-15	100-25	All doses ineffective; tumor growth similar to controls
Vinblastine, q7d × 5	0.8-0.2	Highest dose, T/C >125%; 9 mice surviving; tumors generally smaller than in controls until treatment ceased
Cyclophosphamide, q7d × 5	50-12.5	All doses ineffective; tumors smaller than in controls during period of drug treatment
Methyl-CCNU, q7d × 5	24-6	All doses effective; 16 of 26 survivors have no palpable tumors; animals appear generally healthy
BCNU, q7d × 5	24-6	Highest dose toxic; 19/20 mice surviving at lower doses; 16/19 have no palpable tumors; all appear very healthy

*MST for controls (30 mice) was 40.0 days.

study. Toxicity was evidenced at the highest dose used with each compound, 24 mg/kg/injection. At the two lower doses of methyl-CCNU and BCNU, 39 of 40 mice were still alive at Day 50. In addition, 26 of these mice had no palpable tumor. The BCNU mice appear to be in good health as do those treated with methyl-CCNU at 12 mg/kg/injection. Whether the response to these two nitrosoureas will prove to be greater than that found by Hrushesky and Murphy with CCNU remains to be determined.

CONCLUSIONS

The renal cell carcinoma animal model developed and characterized in BALB/c mice by Murphy and Hrushesky (9) may prove to be a useful system for the selection of chemotherapeutic agents with activity against a slow-growing solid tumor of renal cell origin. The tumor can be transplanted by a variety of routes and drug effects can be measured on tumor size, extent of metastases, and survival time. The ir system has the advantages of a slow-growing solid tumor of specific renal origin, a system which mimics the human disease state including predictable metastases, and as a model using a bilateral organ, it is an ideal situation for surgical adjuvant studies. In addition, the 45-day average MST is workable, allowing for the initiation of therapy at various disease stages. Whether the spectrum of drug activity in this model system will result in information that can be used in the development or selection of drugs for treatment of human clinical disease cannot be foretold from the limited drug trials that have been conducted to date.

LIST OF COMPOUNDS

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8
BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-
Bleomycin: NSC-125066; [2,4'-bithiazole]-4-carboxylic acid, 2'-(2-aminoethyl)-, monohydrate
CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate
Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1
Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-
Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

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Ridgway Osteogenic Sarcoma—A Promising Laboratory Model for Special Therapeutic Trials Against an Advanced-Staged, Drug-Sensitive Animal Tumor System[1]

W. R. Laster, Jr.[2,3]

With the possible exception of acute leukemia, most of the screening and secondary evaluation of potential anticancer agents is done in animal tumor systems that lack an objectively demonstrated correlation with the drug response of any specific type of human neoplasm.

Even though there is this lack of specific correlation, animal tumor models have been useful in selecting numbers of anticancer agents that have been proven to be useful in clinical oncology. Similarly, chemotherapeutic principles, such as therapeutically potentiating drug combinations which are useful in the human disease, have also been developed using animal model systems. The search for better animal tumor models continues and the hope of all experimental oncologists is that one or more systems can be developed that will allow specific correlations to be made with one or more tumors in man.

Old-time friends, such as Sarcoma 180, Carcinoma 755, L1210 leukemia, P388 leukemia, Lewis lung carcinoma, etc, have been and continue to be extremely useful to the experimental chemotherapist, but each has its own weaknesses as well as its good qualities. At Southern Research Institute we believe that the Ridgway osteogenic sarcoma (ROS) will become a valuable addition to the experimental oncologist's armamentarium.

I would like to briefly describe the history and biology of this tumor and then present data that hopefully will show that ROS is an excellent tumor system, particularly for use in the secondary evaluation of anticancer agents.

ROS was first observed in November 1948 by Dr. J.H. Burchenal, at the Sloan-Kettering Memorial Hospital and Tumor Institute in New York, as a spontaneously appearing inguinal mass in a male AKm mouse (1). He gave the tumor-bearing mouse to Dr. D.A. Karnofsky, who established the transplantable tumor in his laboratory. Dr. Karnofsky named it "Ridgway" for Lois Ridgway, the technician in his laboratory who carried out this early work. Histologically, the original spontaneous tumor was made up of solidly packed round cells with foci of bone formation, but after a few sc passages in AKm mice it lost bone structure but retained high alkaline phosphatase activity (2).

We obtained the ROS in September 1972 from Miss Barbara Brown, Department of Pharmacology, Sidney Farber Cancer Center, Boston, Mass. It is being maintained in serial passage in the AKR mouse by sc trocar implant of about 200-mg fragments from a 20-day-old transplanted tumor. In our hands, this tumor is essentially uniformly fatal in the AKR mouse or the F_1 hybrid of the AKR mouse ($AKD2F_1$ or $D2AKF_1$), having a <1% spontaneous regression rate in the AKR/J mouse and a <0.1% spontaneous regression rate in the $AKD2F_1$ hybrid.

At present, all of our chemotherapeutic trials are being done using the $AKD2F_1$ hybrid, a first-generation cross between an AKR female and a DBA/2 male. This hybrid is a much more vigorous animal than the AKR mouse and, in our opinion, is more suited for stressful chemotherapy than the inbred line of origin, the AK mouse.

The tumor growth characteristics of typical ROS tumors in the inbred and hybrid hosts are shown in figure 1. As you will note in the $AKD2F_1$ mouse, the median time is 10 days to 0.5 g, 11 days to 1 g, and 18 days to 5 g, with the ranges shown at each level. The tumor growth is somewhat slower in the AKR mouse, with the medians for 0.5, 1.0, and 5.0 g being 11, 14, and 20 days, respectively. These plots are made up of individual tumor measurements and are representative of what we have observed over the past few years. The median lifespan of ROS-implanted mice is about 25-35 days.

We believe that a great deal of importance should be placed on individual tumor measurements rather than just using mean or median tumor sizes. Figure 2 is an example of what I mean. This figure shows both the individual tumor sizes and median tumor sizes of a group of seven mice bearing ROS ranging from about 350 mg to slightly above 1000 mg at the time of initiation of therapy with palmO-ara-C given at 10 mg/kg, ip, on Days 12-26 after tumor implant. If one only used the median tumor size, one would conclude that the agent was active, based on an increase in lifespan (70 days, with a range of 36-73 days, as compared to a range of 25-35 days in un-

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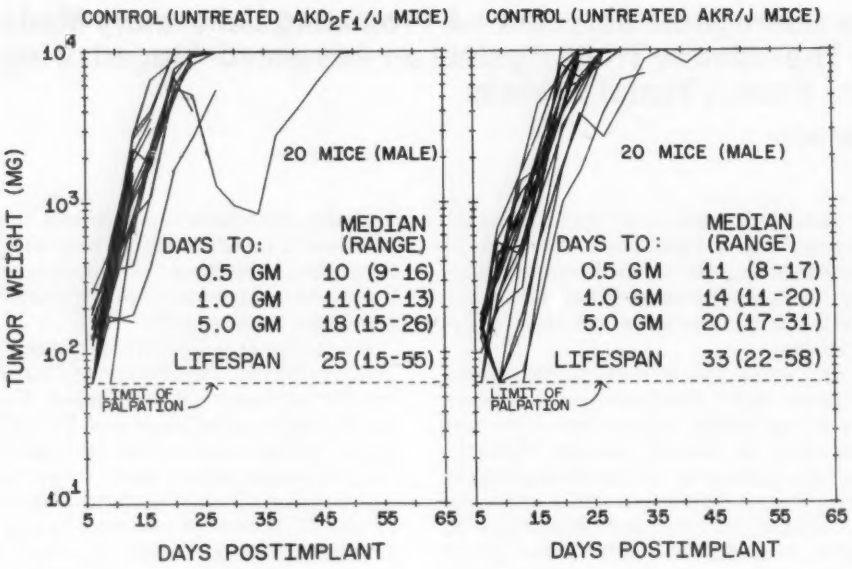

FIGURE 1.—Growth characteristics of ROS in hybrid and inbred mice.

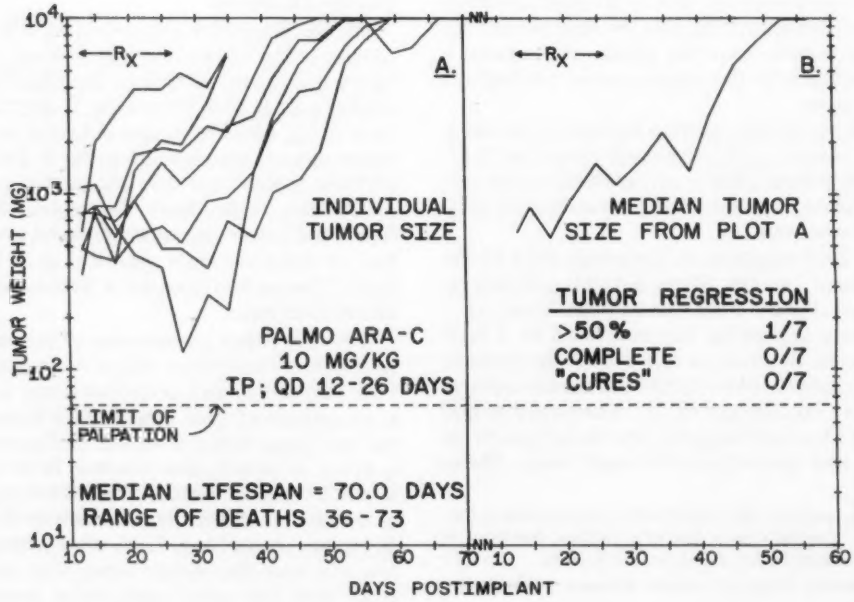

FIGURE 2.—Comparison of individual and median tumor measurements of ROS treated with palmO-ara-C.

treated controls), but that it showed very little tumor regression. However, upon looking at the individual tumor measurements, one sees that the actual response to therapy with the drug among the various tumors is very different. Two of the tumors regressed markedly, with the largest one regressing to almost a tenth of its size at the start of treatment. One tumor did not respond at all, and the other four showed varying degrees of tumor inhibition.

However, this type of variable response does not detract from the usefulness of the ROS. In clinical oncology, physicians do not require that all of a particular type of tumor regress before they consider the candidate antitumor agent to be effective. In fact, treatment of a human cancer resulting in a 20% partial regression rate that formerly had not responded to any treatment at all is considered a therapeutic advance. The clinician then tries to increase the 20% partial regression rate to 40%, 60%, 80%, and 100%. As the partial regression rate approaches 100%, he can expect to begin to see higher complete regression rates, and as these become significant he can hopefully anticipate "cures" and probably normal life expectancies. Based on this, I believe that experimental cancer chemotherapists should also observe individual tumor responses to take advantage of low-incidence partial regressions with the thought of improving activity by schedule changes or with combination therapy with two or more agents.

There are several indications for using the ROS as a tool in experimental cancer chemotherapy. One of these is the fact that ROS will visibly regress and/or be cured by treatment with a number of single drugs that are currently used in effective, but usually noncurative, treatment of a variety of solid tumors of man. As such, this allows model experimental chemotherapy trials with clinically useful drugs using partial regression, complete regression, duration of complete regression, and cures as end points for comparative drug evaluation. These are similar to the end points used by clinical oncologists in evaluating response of most human tumors to treatment.

Eleven agents in wide use and of variable utility against a variety of human neoplasms cause regression of significant percentages of ROS. These include drugs that bind to, intercalate with, or cause breakage of DNA, some classic and useful alkylating agents, purine and pyrimidine antagonists, inhibitors of DNA polymerase, and mitotic inhibitors.

Plotted regression responses of advanced ROS (250 mg to slightly >1 g) to several agents that are classified generally as DNA binders are shown in figure 3. All therapy data presented (unless otherwise noted) were obtained at drug doses that were ≤ the LD10 in nontumor-bearing toxicity control mice that were a part of each experiment. These mice were of the same sex, strain, and source as the tumor-bearing, drug-treated groups.

As will be noted in plot A of figure 3, actinomycin D produced marked activity against established ROS, with eight of eight complete regressions and seven of eight cures. This is not an original observation, as the original work with this agent versus ROS was reported by Sugiura and associates (2), and was expanded by Schwartz and associates (3).

The other DNA binders shown are somewhat less active against ROS than is actinomycin D. In plot B, adriamycin produced seven of eight complete regressions but no cures. Daunorubicin (plot C) produced >50% regressions in nine of 12 mice; three of 12 had complete regressions but, again, no cures. Bleomycin (plot D) was the least active of the four DNA binders, with four of ten mice showing >50% regressions but no complete regressions or cures.

The plots in figure 4 show the response of ROS to a pyrimidine antagonist (5-fluorouracil; 5-FU), a purine antagonist (6-mercaptopurine; 6-MP), an inhibitor of DNA polymerase (palmO-ara-C; the 5′-palmitate depot form of ara-C), and a mitotic inhibitor (vincristine). A high incidence of ≥ 50% regression was obtained with all four agents and complete regressions varying from 40% to 80% were seen with all but 6-MP. No cures were obtained with any of these four compounds. To my knowledge, no other solid animal tumor will regress consistently with 5-FU, 6-MP, or palmO-ara-C therapy. Van Putten has reported regression of an established rhabdomyosarcoma in rats with vincristine (4).

The response of ROS to two classic, clinically useful alkylating agents and to *cis*-dichlorodiammineplatinum(II) (which may or may not be considered an alkylating agent) is shown in figure 5. With cyclophosphamide and melphalan, a high complete regression rate and a significant cure rate were obtained. With *cis*-dichlorodiammineplatinum(II), only two of eight mice had a >50% regression, with no complete regressions. Regression of established ROS has not been obtained with other alkylating agents such as DTIC, BIC, mitomycin C, chlorambucil, thioTEPA, or the nitrosoureas, BCNU, CCNU, or methyl-CCNU.

Another characteristic of ROS that makes it suitable for experimental cancer chemotherapy is that it shows classic and logical dose response to active drugs. Orderly and reproducible dose response is an essential characteristic of an animal tumor model if it is to be used to evaluate agents with potential clinical usefulness. The incidence of partial and/or complete regressions and the median lifespan must decrease as drug dosage decreases. The ability of ROS to follow dose response is shown in figures 6-8.

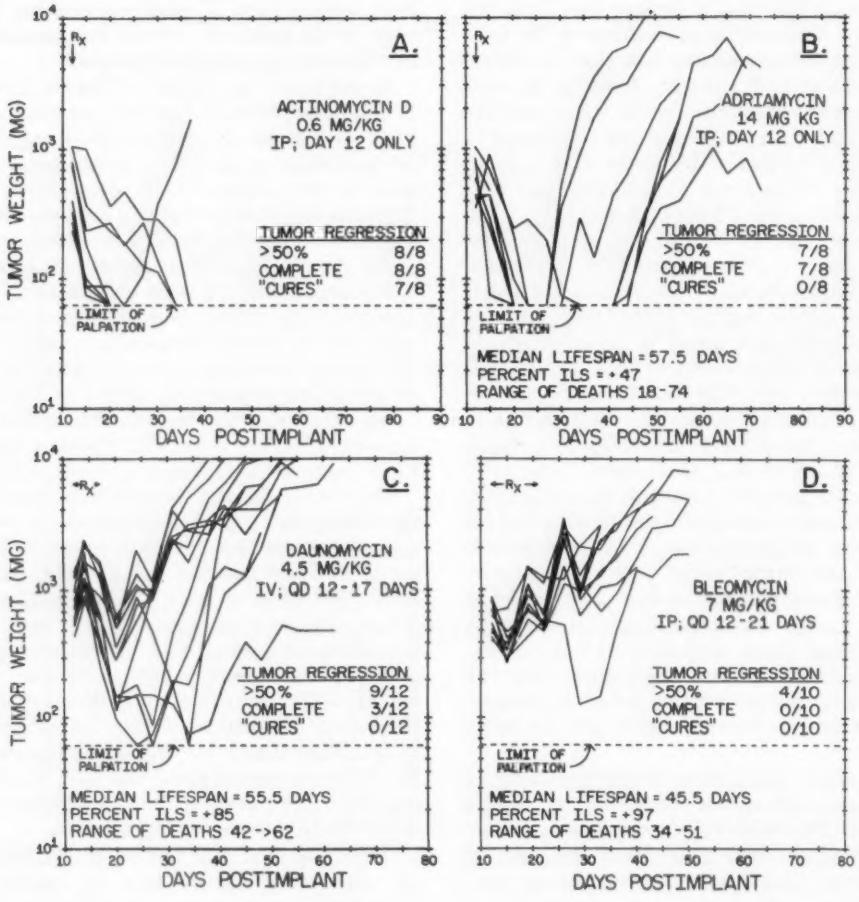

FIGURE 3.—Effect of DNA binders on ROS.

Figure 6 shows the dose response of ROS to decreasing doses of 6-MP given on an 11-day treatment schedule, figure 7 shows a similar study with 5-FU, and figure 8 is a dose response study with cyclophosphamide.

Another indication for using the ROS is the ability to stage to progressively more advanced disease before the initiation of treatment, thus allowing another simulation of comparison with responses of human tumors to treatment. This fact is shown in figures 9 and 10. Using identical doses of each agent given at different times, regression and cure rates are reduced when the tumor mass, at time of initiation of treatment, is increased.

In figure 9 (plot A) with actinomycin D given on Day 12 when the tumor size is about 0.5-1 g, a 100% complete regression rate with seven of eight cures was obtained. However, when therapy was delayed until Day 18 (plot B) when the tumors were 2+ g in size, no complete regressions or cures were obtained. Similar results were obtained with adriamycin (fig 9, plots C and D), 5-FU (fig 10, plots A and B), and melphalan (fig 10, plots C and D). Some may say that this is really what one would logically expect, but this fact is not real until shown to be.

The data presented so far indicate that ROS responds to a number of clinically useful drugs; it responds logically and orderly to decreasing doses of these agents and can be effectively staged for chemotherapeutic trials. Therefore, meaningful experiments can be designed and carried out with combinations of clinically useful drugs by staging the disease beyond the maximum effectiveness of either agent alone at start of therapy. Also, with this type of study, one may be able to answer questions in the laboratory related to the proper sequence of two-

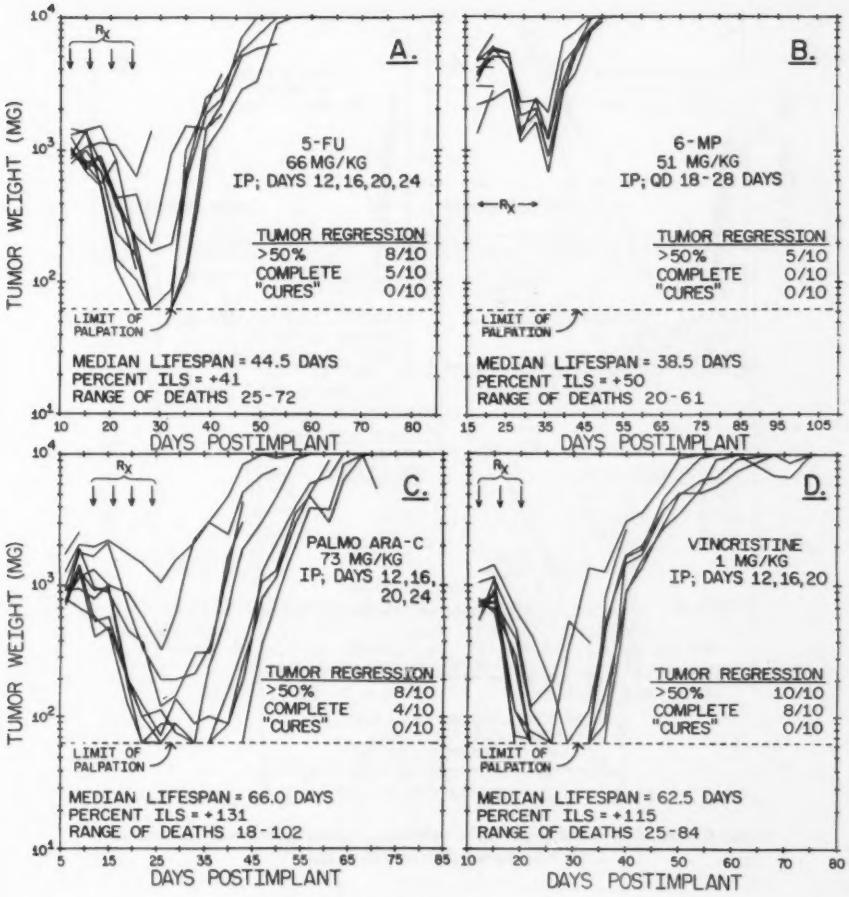

FIGURE 4.—Effect of 5-FU, 6-MP, palmO-ara-C, and vincristine on ROS.

drug combinations, optimal spacing of courses, and whether or not sequential or simultaneous combinations are optimal.

Combination chemotherapy has the promise of being extremely useful by producing less than additive toxicity to vital cells, by potentiating activity against tumor cells, and by destroying tumor cells resistant to one of the agents in the combination. We believe that ROS may be suitable for selecting drug combinations, along with their proper scheduling, that could be useful to the clinician planning new treatment protocols.

Figures 11-16 present some two-drug combinations we have tested in ROS. Therapy with actinomycin D alone, cyclophosphamide alone, or the combination of the two, was started on Day 18 after tumor implant when the tumors were between 1 and 3 g in size. The data presented in figure 11 are the optimal results for each agent alone and in combination. As can be seen in figure 11 (plot B) no complete regressions or cures were obtained with actinomycin D alone; six of eight had >50% regression. With cyclophosphamide alone, seven of eight had >50% regression and one of eight had complete regression. With the optimal doses and schedules (plot F) of cyclophosphamide on Day 18 followed by actinomycin D on Day 25, eight of eight complete regressions and five of eight cures were obtained. A somewhat lesser response was seen when the sequence of drug administration was reversed (plot E); when the two agents were given simultaneously (plot D), no therapeutic potentiation occurred.

Figure 12 shows the results of cyclophosphamide and actinomycin D when given sequentially for four

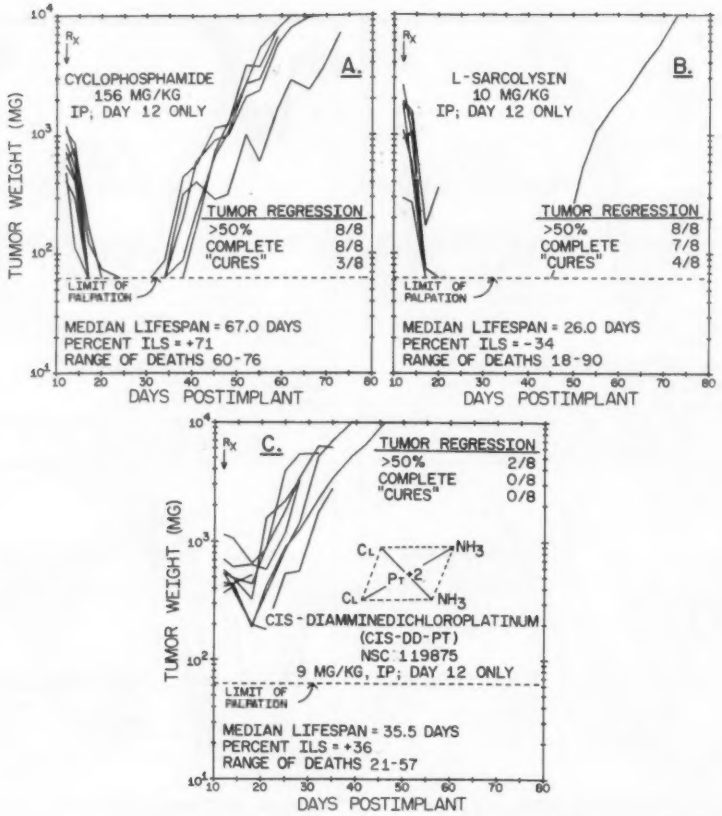

FIGURE 5.—Effect of cyclophosphamide, melphalan (L-sarcolysin), and *cis*-dichlorodiammineplatinum(II) on ROS.

courses. In plot B, cyclophosphamide was given at 100 mg/kg, every 14 days × 4, starting on Day 18 (Days 18, 32, 46, 60), with ten of ten >50% regressions and three of ten complete regressions but no cures. With actinomycin D given at 0.3 mg/kg at the same schedule we had five of ten >50% regressions and one of ten complete regressions but no cures. With the combination where cyclophosphamide was given every 14 days × 4 starting on Day 18 and actinomycin D was given every 14 days × 4 starting on Day 25, we had ten of ten >50% regressions, nine of ten complete regressions, and seven of ten cures. Much more rapid and steady tumor regression occurred under repeated sequential treatment with the combination than with either agent used alone.

Adriamycin plus cyclophosphamide has been reported to be therapeutically synergistic against a number of transplantable solid animal tumors (four lines of C3H mammary carcinomas and B16 melanomas), L1210, and advanced breast tumors in women (5,6). We studied this combination against advanced-staged ROS in a study similar to that with cyclophosphamide plus actinomycin D. As will be noted in figure 13 (plots D, E, and F), clear therapeutic synergism was observed, but in this combination no difference in results was obtained between simultaneous and sequential (either order) therapy.

Drs. Goldin and Johnson and associates have observed therapeutic synergism between cyclophosphamide and melphalan against L1210, and we have confirmed their observation (7,8). Since both cyclophosphamide and melphalan are so active in causing regression of established ROS, we evaluated this combination against ROS staged beyond single-dose cure with either agent alone. In this experiment, each agent alone was given on Day 18 after tumor implant and the combination was given simultaneously on Day 18 or sequentially on Days 18 and 25. As can be seen in figure 14, therapeutic potentiation, based on an increased complete regression rate, was

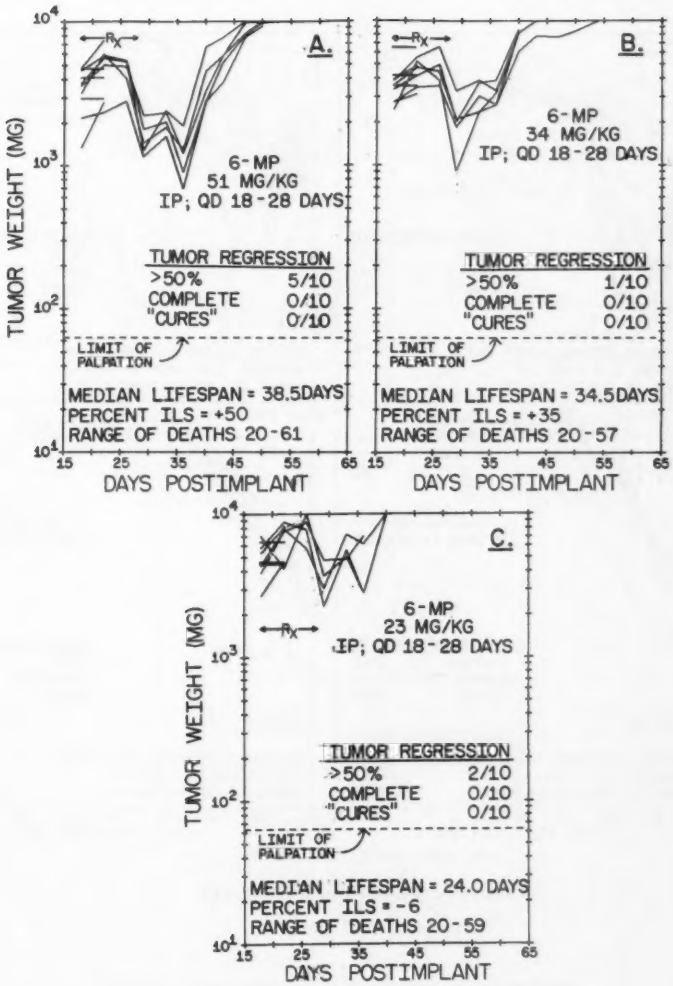

FIGURE 6.—Dose response of ROS to 6-MP.

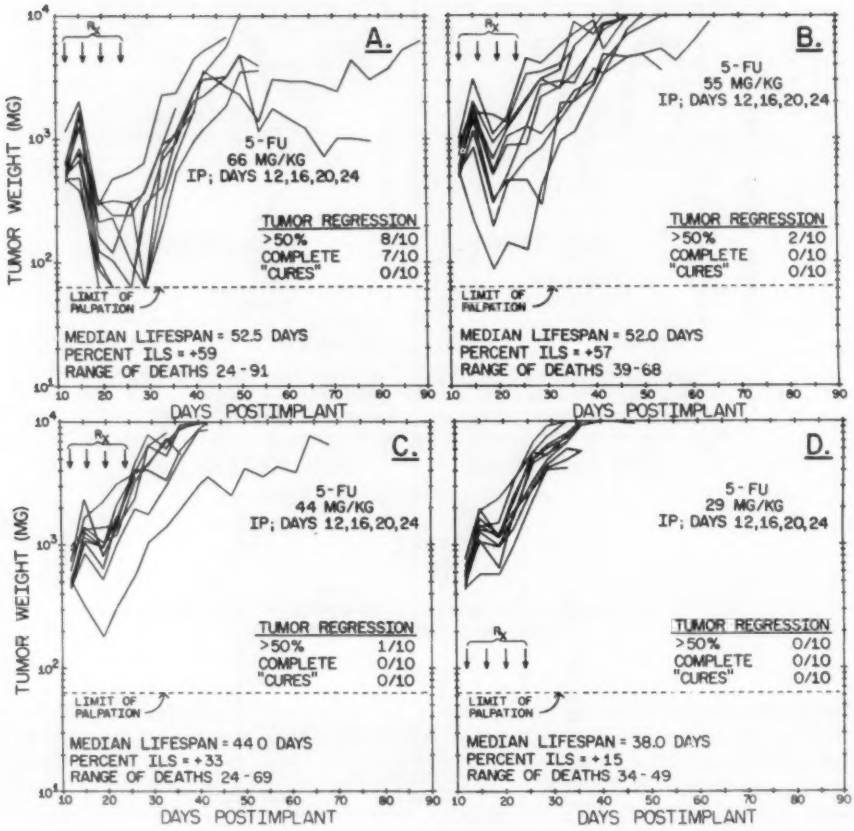

FIGURE 7.—Dose response of ROS to 5-FU.

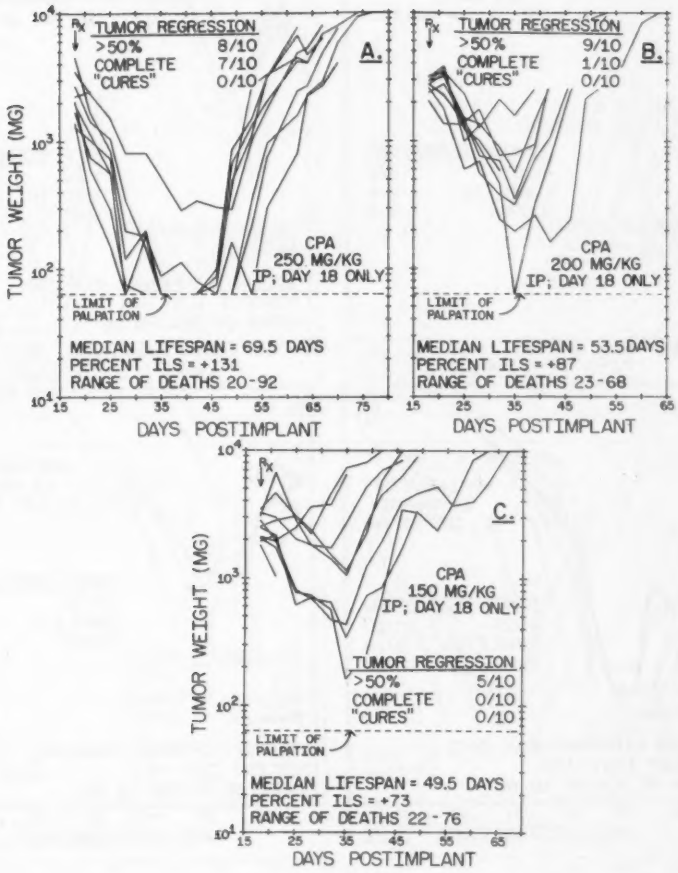

FIGURE 8.—Dose response of ROS to cyclophosphamide (CPA).

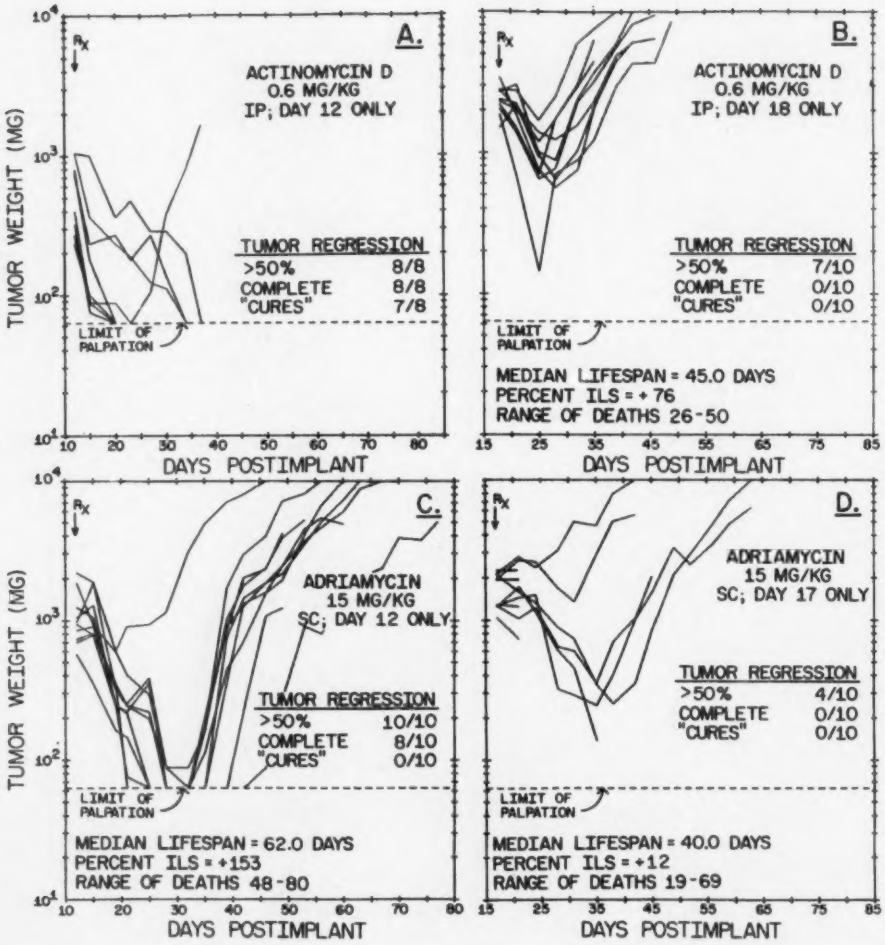

FIGURE 9.—Clinical staging and response of ROS to actinomycin D and adriamycin.

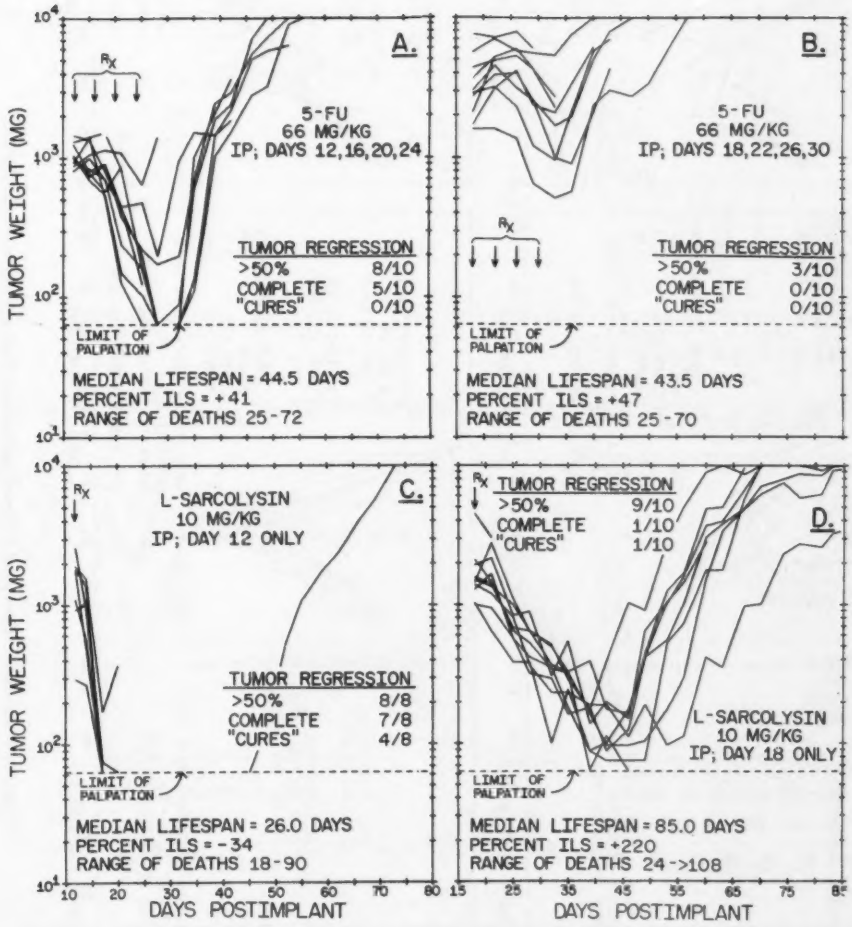

FIGURE 10.—Clinical staging and response of ROS to 5-FU and melphalan (L-sarcolysin).

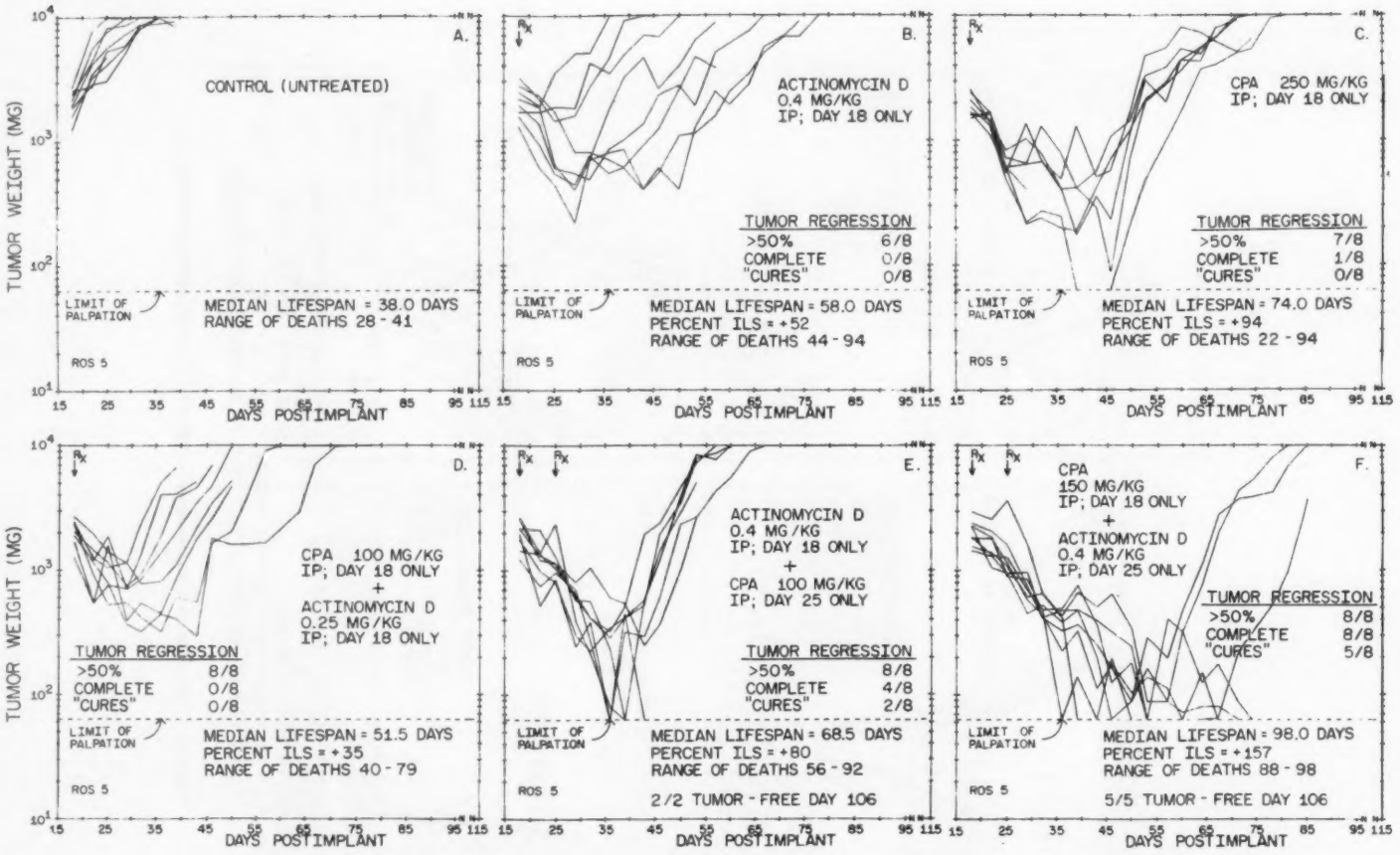

FIGURE 11.—Combination therapy of ROS with actinomycin D plus cyclophosphamide (CPA).

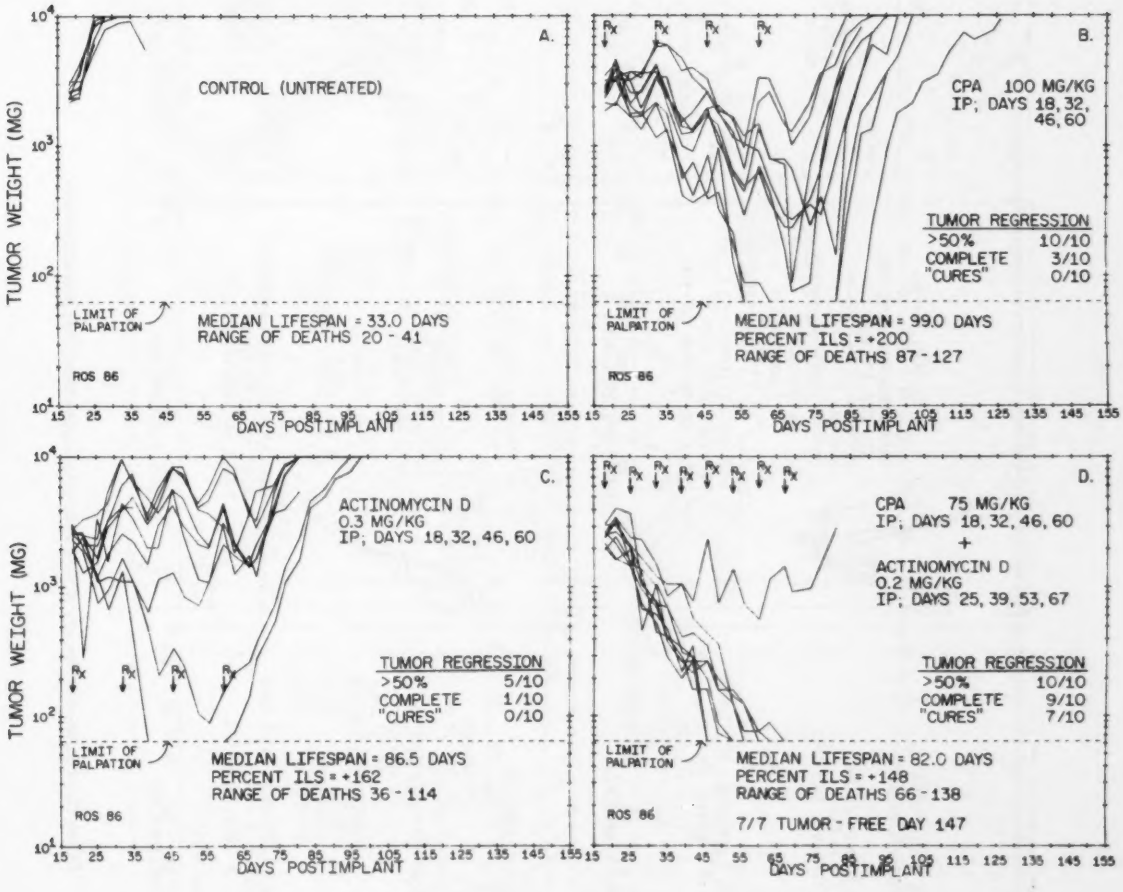

FIGURE 12.—Combination therapy of ROS with cyclophosphamide (CPA) plus actinomycin D given for 4 courses.

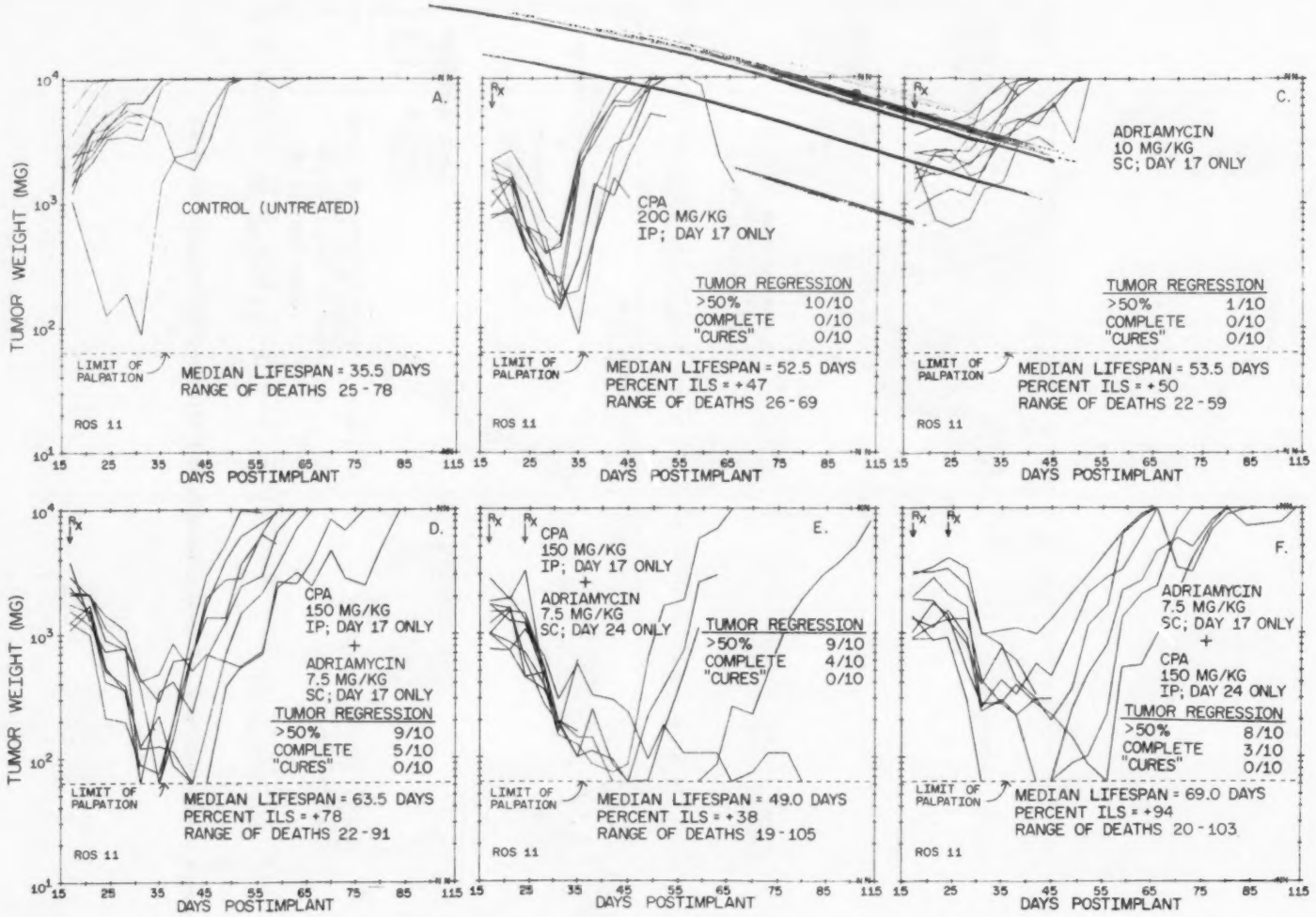

FIGURE 13.—Combination therapy of ROS with cyclophosphamide (CPA) plus adriamycin.

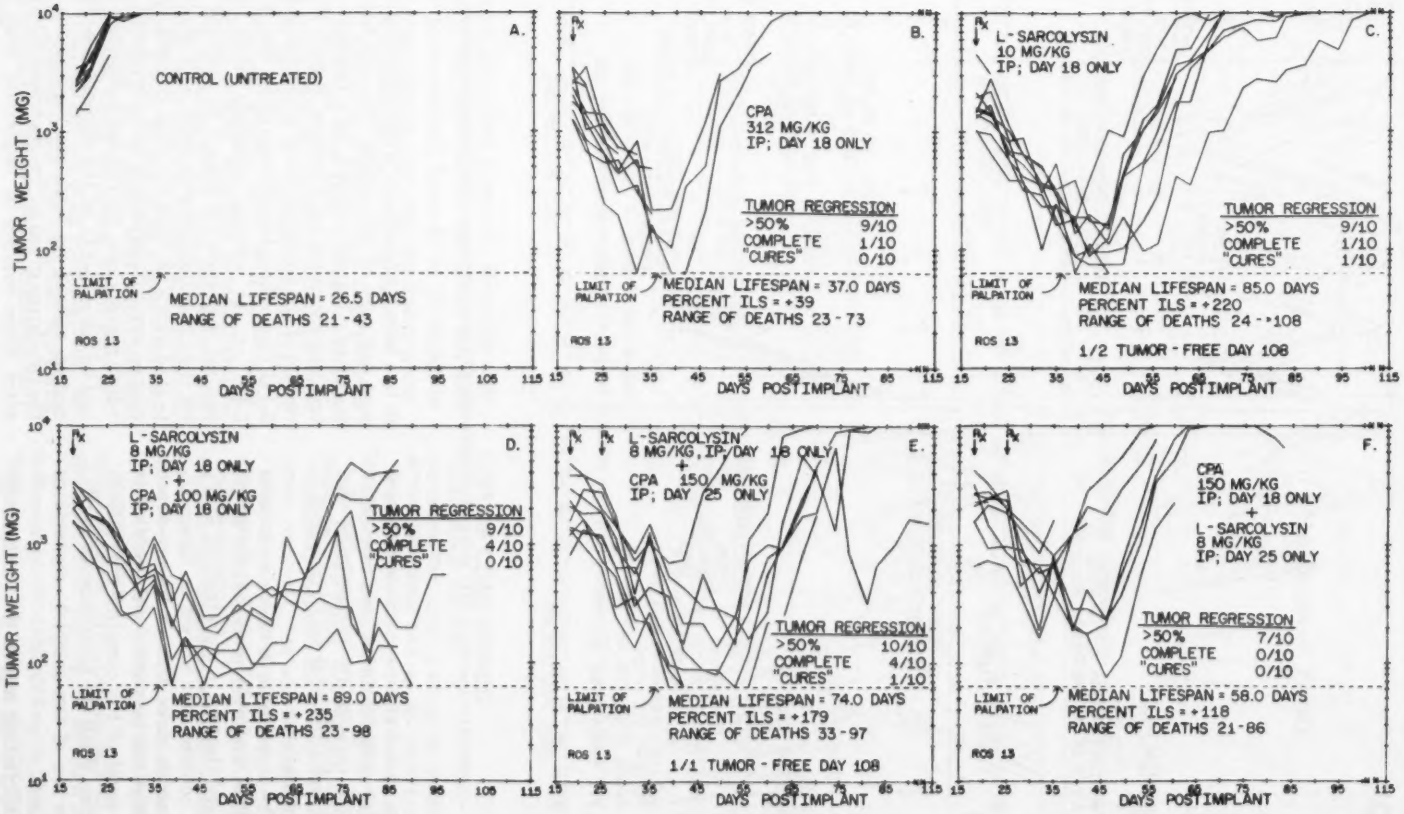

FIGURE 14.—Combination therapy of ROS with cyclophosphamide (CPA) plus melphalan (L-sarcolysin).

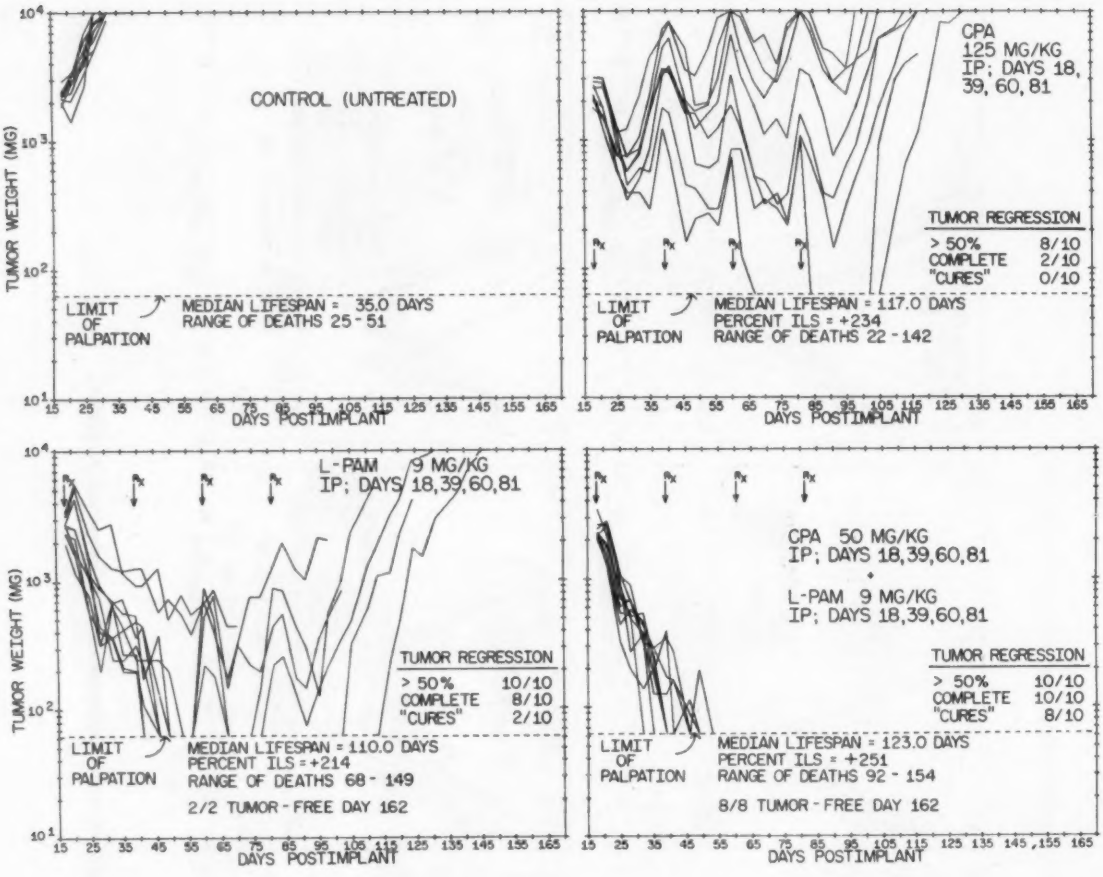
CONTROL (UNTREATED)
LIMIT OF PALPATION
MEDIAN LIFESPAN = 35.0 DAYS
RANGE OF DEATHS 25 - 51
TUMOR WEIGHT (MG)
DAYS POSTIMPLANT
CPA 125 MG/KG IP; DAYS 18, 39, 60, 81
TUMOR REGRESSION
> 50% 8/10
COMPLETE 2/10
"CURES" 0/10
MEDIAN LIFESPAN = 117.0 DAYS
PERCENT ILS = +234
RANGE OF DEATHS 22 - 142
L-PAM 9 MG/KG IP; DAYS 18,39,60,81
TUMOR REGRESSION
> 50% 10/10
COMPLETE 8/10
"CURES" 2/10
MEDIAN LIFESPAN = 110.0 DAYS
PERCENT ILS = +214
RANGE OF DEATHS 68 - 149
2/2 TUMOR - FREE DAY 162
CPA 50 MG/KG IP; DAYS 18,39,60,81
L-PAM 9 MG/KG IP; DAYS 18,39,60,81
TUMOR REGRESSION
> 50% 10/10
COMPLETE 10/10
"CURES" 8/10
MEDIAN LIFESPAN = 123.0 DAYS
PERCENT ILS = +251
RANGE OF DEATHS 92 - 154
8/8 TUMOR - FREE DAY 162

FIGURE 15.—Combination therapy of ROS with cyclophosphamide (CPA) plus melphalan (L-PAM) given in 4 courses.

observed with the simultaneous combination and with the sequential combination where melphalan was given first. No therapeutic potentiation was observed when cyclophosphamide was given first in the sequential combinations.

The results of the experiment in which we treated advanced-staged ROS with cyclophosphamide alone, melphalan alone, and the two in combination, every 21 days for four courses, are shown in figure 15. With cyclophosphamide alone, there were two of ten complete regressions and no cures; with melphalan alone, there were eight of ten complete regressions and two of ten cures. With the simultaneous combination, there were ten of ten complete regressions and eight of ten cures.

The concept of alkylating agents plus antimetabolites in sequential treatment with repeated courses has been successful in producing complete regression and cure of Carcinoma 755, and has been proposed as a logical concept, on a theoretic basis, for treating advanced solid tumors. Figure 16 shows the results of the combination of cyclophosphamide plus 5-FU. Cyclophosphamide was given first, followed by 5-FU on its optimal schedule when used alone against ROS (every 4 days × 4), starting 4 days after the cyclophosphamide dose. This course was repeated every 23 days for four courses. On a single course, cyclophosphamide alone produced nine of ten >50% regressions, four of ten complete regressions, and no cures. 5-FU, at the optimal dose for a single course, caused no complete regressions. However, the two drugs given at the maximum tolerated doses, after three courses of sequential drug treatment, gave a 90% partial regression rate, a 70% complete

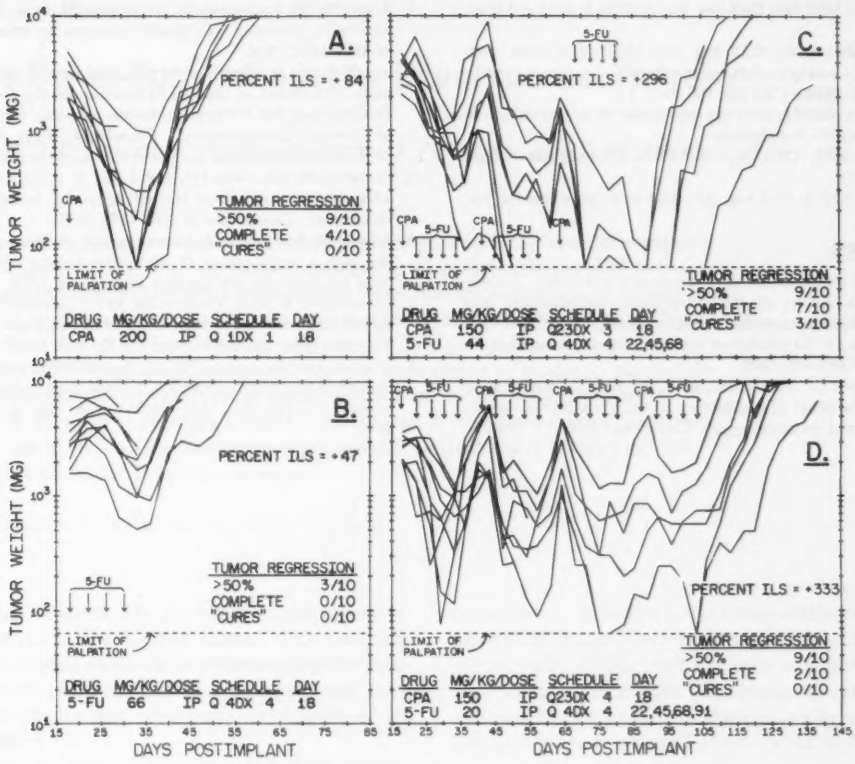

FIGURE 16.—Combination therapy of ROS with cyclophosphamide (CPA) plus 5-FU.

regression rate, and a 30% cure rate. When an additional course of the combination was given, the dose of 5-FU had to be reduced from 44 to 20 mg/kg to avoid lethal drug toxicity, and this resulted in a lower complete regression rate with no cures.

Examples of two-drug combinations that produce therapeutic synergism when used to treat advanced-staged ROS have been described. These data show that ROS can be used to demonstrate synergism between various clinically useful drugs, including optimal sequence and/or schedule determinations. However, I am not suggesting that this tumor will predict the ideal combination, sequence, or schedule for any human tumor to any drug combination described. These data do suggest that experimental tumor systems are being developed which may produce useful information on drug combinations, sequences, and schedules for the clinician designing treatment protocols using presently available anticancer agents.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

BIC: NSC-82196; CAS reg. No. 5034-77-5; imidazole-4-carboxamide, 5-[3,3-bis(2-chloroethyl)-1-triazeno]-

Bleomycin: NSC-125066; [2,4'-bithiazole]-4-carboxylic acid, 2'-(2-aminoethyl)-, monohydrate

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; [*p*-[bis(2-chloroethyl)amino]phenyl]-

***Cis*-dichlorodiammineplatinum(II):** NSC-119875; CAS reg. No. 15663-27-1

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

Daunorubicin: NSC-82151; CAS reg. No. 23541-50-6; daunomycin

DTIC: NSC-45388; CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-; L-sarcolysin

6-Mercaptopurine: NSC-755; CAS reg. No. 6112-76-1; purine-6-thiol, monohydrate

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

Mitomycin C: NSC-26980; CAS reg. No. 50-07-7

PalmO-ara-C: NSC-135962; CAS reg. No. 31088-06-9; cytosine, 1-β-D-arabinofuranosyl-, 5'-palmitate

ThioTEPA: NSC-6396; CAS reg. No. 52-24-4; phosphine sulfide, tris(1-aziridinyl)-

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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A Mouse Colon-Tumor Model for Experimental Therapy[1]

T. H. Corbett, D. P. Griswold, Jr., B. J. Roberts, J. Peckham, and F. M. Schabel, Jr.[2,3]

SUMMARY

Four mouse colon tumors were established in serial passage. The chemotherapeutic responsiveness of three of these tumors appears to be sufficiently parallel to the responsiveness of human colorectal tumors that these tumors may serve as predictive models for combination chemotherapy and combination modality therapies. Two nitrosourea-fluorinated pyrimidine combinations (BCNU plus 5-fluoro-2′-deoxyuridine and methyl-CCNU plus 5-fluorouracil) have been found to be modestly potentiating. These combinations are attractive candidates for surgical/chemotherapeutic adjuvant trials because of their high curative potential. A water-soluble nitrosourea, CCCNU-*cis*, has been found to have very high activity against colon tumors as well as five other transplantable tumors in mice.

[Cancer Chemother Rep Part 2, vol 5:169-186, 1975]

Colon cancer is currently the second leading cause of cancer deaths in the United States. The need for improved treatment methods is evidenced by the fact that the percent of 5-year survivors and the mortality rate from this tumor type have not improved in the last 20 years (1).

Apart from an improvement in detection procedures, well-chosen chemotherapy, especially in conjunction with surgical treatment, probably offers the best potential for improving mortality rates. The selection of this "well-chosen" chemotherapy from an enormous number of possibilities falls partly on the lower-animal experimentalist. Unfortunately, until very recently, the experimentalist has had no suitable model system for this tumor type.

Tumor Induction

Toward this end, we induced and transplanted a large number of colon tumors in mice (2). Four of these tumors were established in serial passage and are currently being used for chemotherapy studies.

The four agents used to induce these colon tumors were 1,2-dimethylhydrazine (DMH), *N*-methyl-*N*-nitrosourea, *N*-methyl-*N*-nitrosourethane, and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. One of the compounds, DMH, and cycasin are metabolized to the same proximate carcinogen, methylazoxymethanol (3). Cycasin is the naturally occurring colon carcinogen isolated from cycad nuts (4). All of

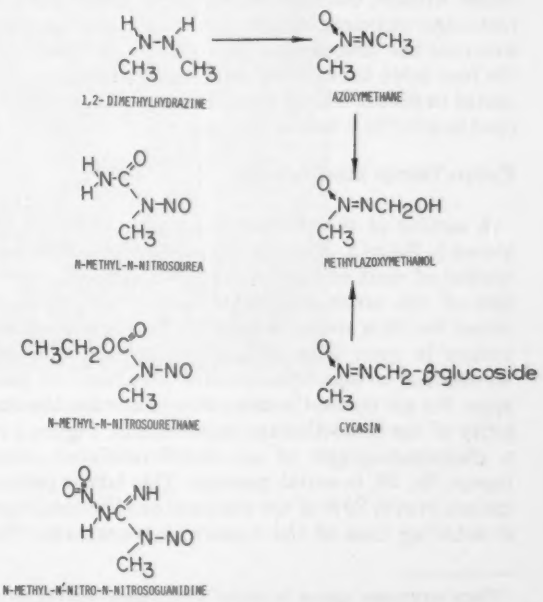

FIGURE 1.—Seven colon carcinogens that have a structural similarity (the-N-N-CH_3 grouping). Cycasin (isolate from cycad nuts) and 1,2-dimethylhydrazine are metabolized in vivo to the same compound, methylazoxymethanol.

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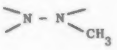
TABLE 1.—Chemical induction of colon tumors in mice

Carcinogen	No. of mice	No. of colon tumors found*	No. of histologically confirmed colon tumors	No. in serial passage
1,2-Dimethylhydrazine	406	39	13	3
N-methyl-*N*-nitrosourethane	200	30	21	1
N-methyl-*N*-nitrosourea	200	17	8	0
N-methyl-*N'*-nitro-*N*-nitrosoguanidine	205	16	6	0
	1011	102	48	4

*Eighty-two colon tumors were transplanted. Thirty-four of the colon tumors were very small and none of the tissue was sacrificed for histology. None of these small tumors survived the first-generation transplant.

the effective colon carcinogens contained a molecusimilarity, an



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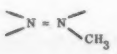


grouping[4] (*see* fig 1). The number of colon tumors induced by each of the four agents is shown in table 1. Eighty-two of the tumors were found in sacrificed mice and were transplanted. Thirty-four of these tumors were small (<80 mg) and were transplanted intact without the sacrifice of any of the tissue for histologic examination. None of these small tumors survived the first-generation transplant. Three of the four colon tumors that survived transplant originated in female Balb/c mice. The other tumor developed in a C57Bl/6 female mouse.

Colon Tumor Morphology

A section of the C57Bl/6 colon tumor, No. 38, is shown in figure 2. It was a polypoid adenocarcinoma, typical of most of the colon tumors induced. A section of the ninth transplant generation of colon tumor No. 38 is shown in figure 3. This tumor metastasizes in more than 40% of the animals and has maintained a well-differentiated structure in passage. We are currently using this tumor for the majority of the chemotherapy experiments. Figure 4 is a photomicrograph of an undifferentiated colon tumor, No. 26, in serial passage. This tumor metastasizes in over 70% of the mice and has the most rapid doubling time of the tumors in transplant. The third colon tumor that is used for chemotherapy studies is shown in figure 5. This tumor is a well-differentiated adenocarcinoma and has the slowest doubling time of the tumors in transplant.

Response to Chemotherapy

We are currently screening a number of antitumor agents against the transplantable colon tumors. The results of a few of these drug trials against small tumor implants are shown in tables 2-4. An arbitrary activity rating was assigned to these compounds based on T-C,[5] %ILS,[6] and tumor-free survivor values (table 5). In general, if an agent or class of agents was effective against one of the colon tumors, it was also active against the others, with some variations in the degree of responsiveness (*see* tables 3-5). Three of the agents, palmO-ara-C, actinomycin D, and dianhydrogalactitol, were adequately tested against only one of the colon tumors.

Measurements of Antitumor Activity

Different end points were used to assess antitumor activity than are commonly used in the clinic. Delay in tumor growth, %ILS, and cures of animals bearing small tumor implants were used to measure antitumor activity. None of the compounds listed as a plus one (+) in table 5 are sufficiently active in the schedules that were used to effect regressions greater than 50% in far-advanced tumors (one of the clinical criteria of antitumor activity).

[4]These structures appear to confer carcinogenic activity on a compound. Three commonly used antitumor agents contain this structure: DTIC (NSC-45388), procarbazine (NSC-77213), and streptozotocin (NSC-85998). All three compounds are highly carcinogenic (5,6).

[5]T–C (days) = the time required for the treatment-group tumors (median of ten) to reach 750 mg, minus the time required for the control-group tumors (median of ten) to grow to the same size. Tumor-free survivors are excluded from the median tumor-weight calculations.

[6]Percent increase in lifespan (%ILS) = 100 × (the median day of death of the treated-group mice, minus the median day of death of the control-group mice, divided by the median day of death of the control group). Tumor-free survivors are excluded from the median day-of-death calculations.

FIGURE 2.—Colon tumor No. 38: original tumor mass (H & E stain, × 70).

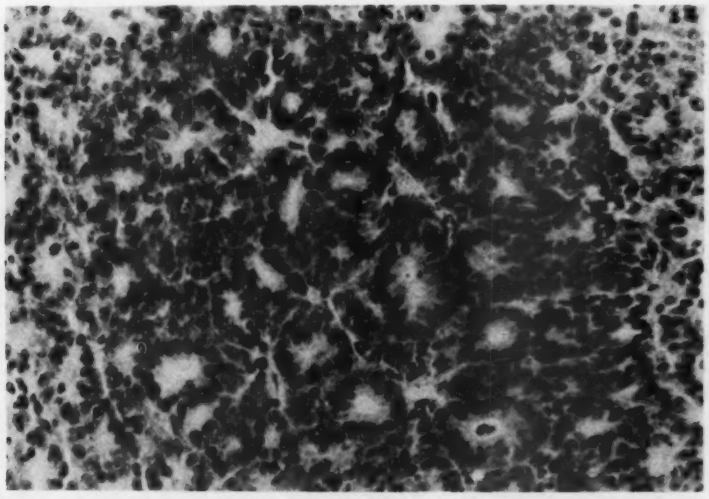


FIGURE 3.—Colon tumor No. 38: 9th transplant generation (H & E stain, × 250).

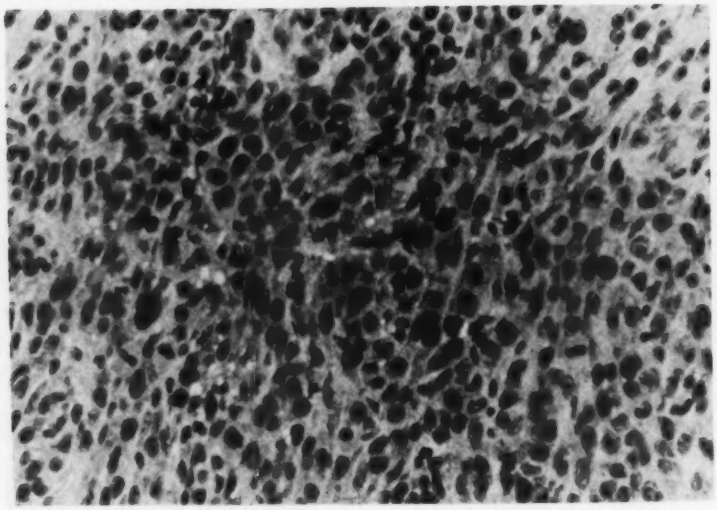


FIGURE 4.—Colon tumor No. 26: 22nd transplant generation (H & E stain, × 250).

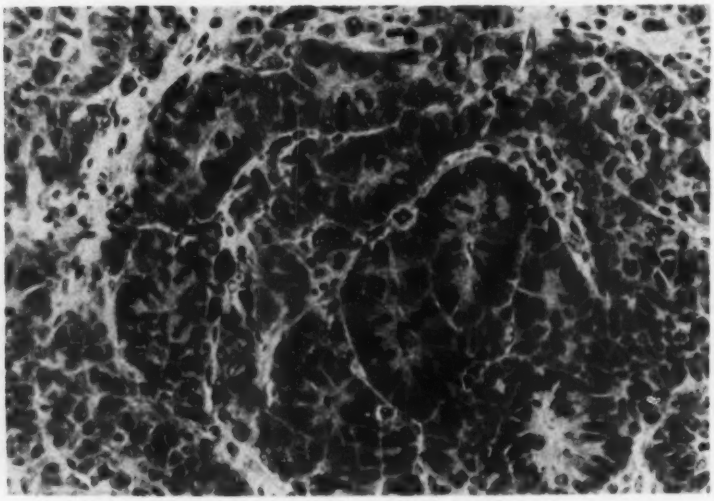


FIGURE 5.—Colon tumor No. 36: 14th transplant generation (H & E stain, × 250).

TABLE 2.—Chemotherapy of colon tumor No. 26

Transplant Generation	Agent	Drug Route	Dosage (mg/kg/dose)	Schedule (days)	Duration of Experiment	No. of Drug Induced Toxicity Deaths/Total	Median Day of Death of Control	Percent Increase in Life Span of Treated Mice[1]	Time to 750 mg of Control	T-C[2] (days)	Tumor-Free Survivors (controls)	Tumor-Free Survivors (treated)
08	CCCNU-trans	iv	15	3	81 days	0/10	35	80	17	25	0/19	5/10
08	MeCCNU	ip	28	3	81 days	0/10	35	88	17	13	0/19	4/10
06	BCNU	ip	24	3	76 days	3/10	51.5	8	17	8	0/20	0/10
05	5-FUdR	sc	110	3-7	65 days	0/10	52.5	5	13	9	0/10	0/10
05	Cyclophosphamide	ip	270	3	65 days	0/10	52.5	2	13	11	0/10	0/10
04	Mitomycin-C	iv	2.2	3,10,17	83 days	2/10	71	7	17	12	0/10	0/10
08	Hexamethylmelamine	ip	95	3-7	81 days	3/10	35	8	17	3	0/19	0/10
06	Dibromodulcitol	ip	120	3-7	84 days	1/10	52.5	14	16	12	0/20	1/10
06	Adriamycin	sc	3.2	3-7	84 days	0/10	52.5	7	16	10	0/20	0/10
06	Amethopterin	ip	1.2	3-7	84 days	0/10	52.5	-4	16	2	0/20	0/10

Method: Trocar fragments, 20 mg each, were implanted in the axillary region of female Balb/c mice on day 0. The mice were randomly distributed between the treatment and control groups. Chemotherapy was started on day 3.

[1]Percent increase in life span = 100 x (median day of death [MDD] of the treated mice, minus the MDD of the control mice) divided by the MDD of the controls. Tumor-free survivors were excluded from the calculations.

[2]T-C (days) = time required for the treatment group tumors (median of 10) to reach 750 mg, minus the time required for the control group tumors (median of 10) to grow to the same size. Tumor-free survivors were excluded in the median tumor weight calculations. An agent with a T-C value of less than 7 days was considered inactive at the dose, schedule, and route used.

TABLE 3.—Chemotherapy of colon tumor No. 36

Transplant Generation	Agent	Drug Route	Dosage (mg/kg/dose)	Schedule (days)	Duration of Experiment	No. of Drug Induced Toxicity Deaths/Total	Median Day of Death of Control	Percent Increase in Life Span [1] of Treated Mice	Time to 750 mg of Control	T-C (days)	Tumor-Free Survivors (controls)	Tumor-Free Survivors (treated)
05	CCCNU-trans	iv	26	3	144 days	1/10	93.5	*	35	52	0/10	8/10
15	MeCCNU	iv	42	3	75 days	0/10	48.5	51	21	4	0/10	1/10
05	BCNU	iv	36	3	144 days	1/10	90	34	40	14	1/20	3/10
15	5-FU	iv	44	3,10	75 days	0/10	48.5	29	21	10	0/10	1/10
05	5-FUdR	sc	110	3-7	80 days	0/10	93.5	-9	35	5	0/10	1/10
05	Cyclophosphamide	ip	180	3	80 days	1/10	93.5	32	35	23	0/10	3/10
15	Mitomycin-C	iv	3.3	3,10	75 days	2/10	48.5	27	21	16	0/10	0/10
05	Dibromodulcitol	ip	120	3-7	81 days	0/10	85	40	47	11	5/20	3/10
05	Adriamycin	sc	3.2	3-7	81 days	0/10	85	108	47	0	0/20	5/10
05	Vinblastine	iv	6.0	3	81 days	1/10	90	27	40	13	1/20	2/10
05	Amethopterin	ip	2.8	3-7	81 days	0/10	85	31	47	4	5/20	1/10

Method: Trocar fragments, 20 mg each, were implanted in the axillary region of female Balb/c mice on day 0. The mice were randomly distributed between the treatment and control groups. Chemotherapy was started on day 3.

[1,2]See footnotes 1 and 2, Table 2.

*Excessive numbers of cures (8/10) prevent determination of a meaningful % ILS value.

TABLE 4.—Chemotherapy of colon tumor No. 38

Transplant Generation	Agent	Drug Route	Dosage (mg/kg/dose)	Schedule (days)	Duration of Experiment	No. of Drug Induced Toxicity Deaths/Total	Median Day of Death of Control	Percent Increase in Life Span of Treated Mice[1]	Time to 750 mg of Control	T-C[2] (days)	Tumor-Free Survivors (controls)	Tumor-Free Survivors (treated)
04	CCCNU-trans	iv	17	3	136 days	0/10	80.5	48	30	14	0/20	1/10
04	MeCCNU	ip	42	3	136 days	0/10	80.5	32	30	14	0/20	1/10
10	5-FU	ip	98	3,10,17,24	113 days	0/10	51	45	22	35	0/10	1/10
04	5-FUdR	sc	110	3-7	136 days	0/10	80.5	32	30	11	0/20	4/10
04	Cyclophosphamide	ip	270	3	136 days	0/10	80.5	40	30	19	0/20	1/10
12	Mitomycin-C	iv	2.2	3,10,17	111 days	2/10	61	57	19	9	0/10	0/10
16	Hexamethylmelamine	po	200	3,10,17,24,31	>66 days	0/10	46.5	29	27	6	0/10	0/10
12	PalmO-ara-C	sc	91	3,10,17,24	68 days	0/10	39	40	18	28	0/10	0/10
19	Dibromodulcitol	ip	300	3,10,17,24	>83 days	0/10	67	22	18	10	1/10	1/10
19	Dianhydrogalactitol	iv	10	3,10,17	56 days	1/10	31.5	44	17	19	0/8	0/10
04	Adriamycin	sc	2.7	3-10	136 days	0/9	80.5	45	30	10	0/20	1/9
13	Vincristine	iv	1.65	3,10,17	59 days	1/10	25	100	18	0	0/10	0/10
04	Vinblastine	iv	6.0	3	136 days	0/10	80.5	21	30	12	0/20	1/10
16	Actinomycin-D	iv	0.6	3,10,17,24,31	>66 days	1/10	46.5	In Progress	27	14	0/10	2/10
19	Amethopterin	iv	38	3,10,17,24	56 days	0/10	31.5	75	17	2	0/8	0/10

*Tested below optimum dosage which is 33-40 mg/kg.

Method: Trocar fragments, 20 mg each, were implanted in the axillary region of female BDF_1 mice on day 0. The mice were randomly distributed between the treatment and control groups. Chemotherapy was started on day 3.

[1,2]See footnotes 1 and 2, Table 2.

TABLE 5.—Chemotherapeutic response of transplantable mouse colon tumors and human colon tumors

	Arbitrary activity rating	
	Colon tumors in mice*	Colon tumors in humans†
CCCNU-*cis*	+++	Unknown
Methyl-CCNU, BCNU	++	++ (8,9,‡)
5-FUDR, 5-FU	++	++ (7,8,9,‡)
Cyclophosphamide	++	+ or ++ (7,8,‡)
Mitomycin C	+	− or + (8,‡)§
Hexamethylmelamine	−	− or + (11,‡)§
Ara-C (palmO-ara-C)	(+)	± (‡)
Dibromodulcitol	+	− or + (12,‡)§
Dianhydrogalactitol	+	Unknown
Adriamycin	+	− or +(8,‡)§
Vinblastine	+	± (13,‡)
Vincristine	−	− (‡)
Methotrexate	−	+ (8,‡)

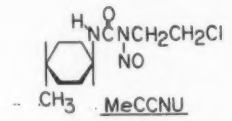
*Antitumor activity against transplantable mouse colon tumors. See tables 2-4 and figures 8, 10, and 11.

†Nos. in parentheses are reference Nos.

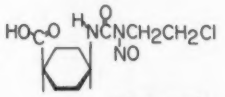
‡Contractor's Report to the DCT, NCI, from Southern Research Institute (contract N01-CM-43756). Combination Chemotherapy and Combination Therapy. Special Report, Booklet 9, 1974.

§Conflicting results.

There was some disagreement in the literature as to the usefulness of various agents against human colorectal tumors (table 5). Fluorinated pyrimidines, cyclophosphamide, and certain nitrosoureas appear to be among the most active agents. In order to determine correlative drug sensitivities between transplantable mouse colon tumors and human colorectal tumors, these three classes of agents have been examined in greatest detail (tables 2-4, and figs 8, 10, and 11).



MeCCNU



CCCNU-cis

FIGURE 6.—Structure of a new water-soluble nitrosourea. CCCNU-*cis* has a carboxylic acid group at the 4 position of the cyclohexane ring in place of the methyl group of methyl-CCNU.

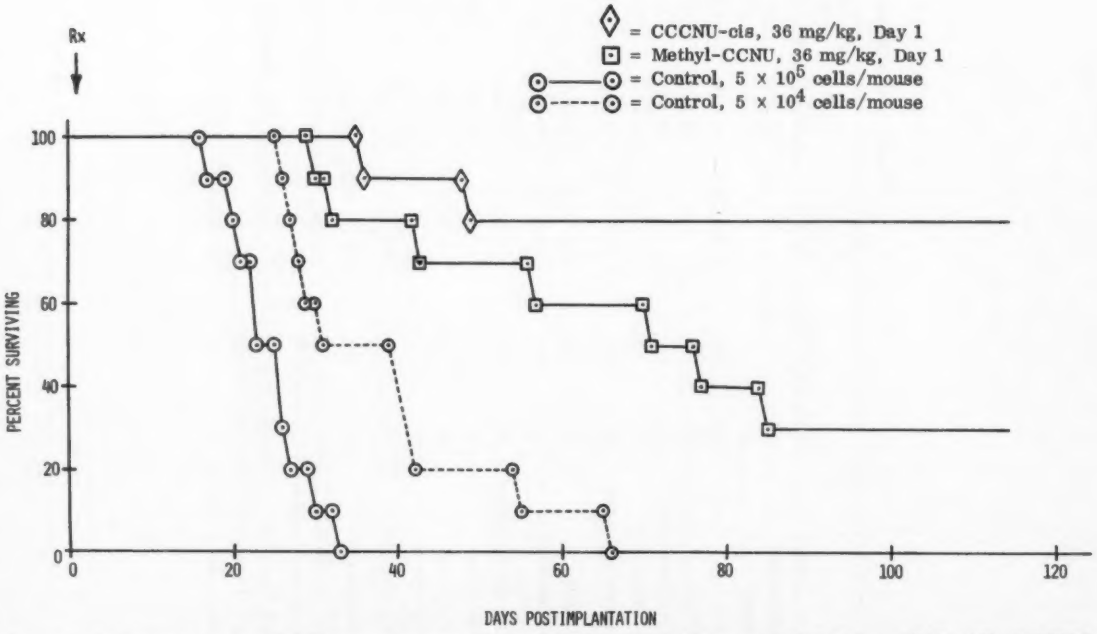


FIGURE 7.—CCCNU-*cis* and methyl-CCNU treatment of mice implanted iv with mammary tumor cells. Titered tumor cells (5×10^5 cells/B6C3F_1 mouse) were implanted iv on Day 0. These mice were randomized and distributed among the various control and treatment groups. An additional group of ten mice was implanted with 5×10^4 cells to indicate the take rate of the tumor. Animals surviving on Day 114 were necropsied and found to be tumor-free.

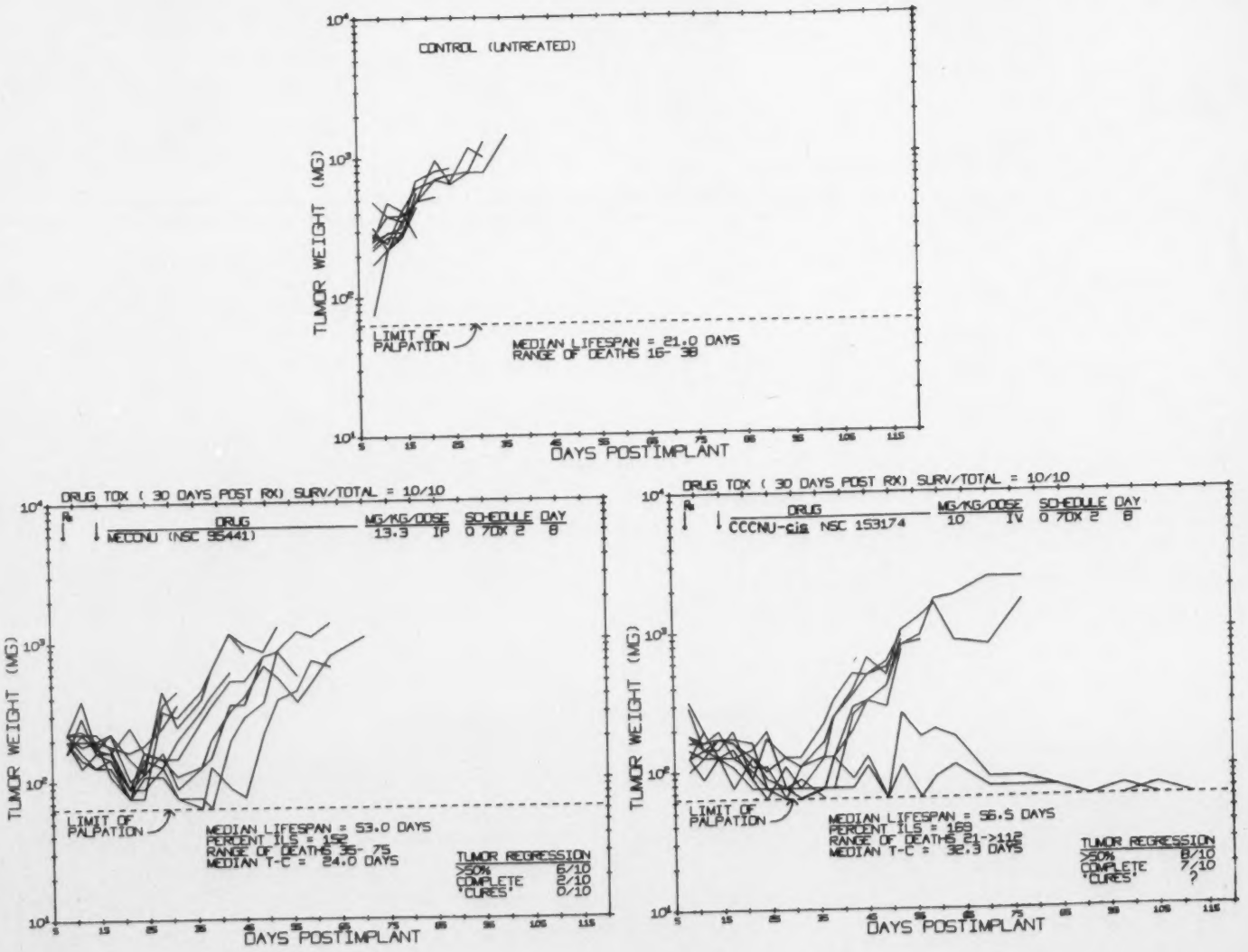


FIGURE 8.—CCCNU-*cis* and methyl-CCNU treatment of advanced colon tumors. Sc growing tumors (No. 26) were allowed to become large (100-400 mg) before treatment was initiated.

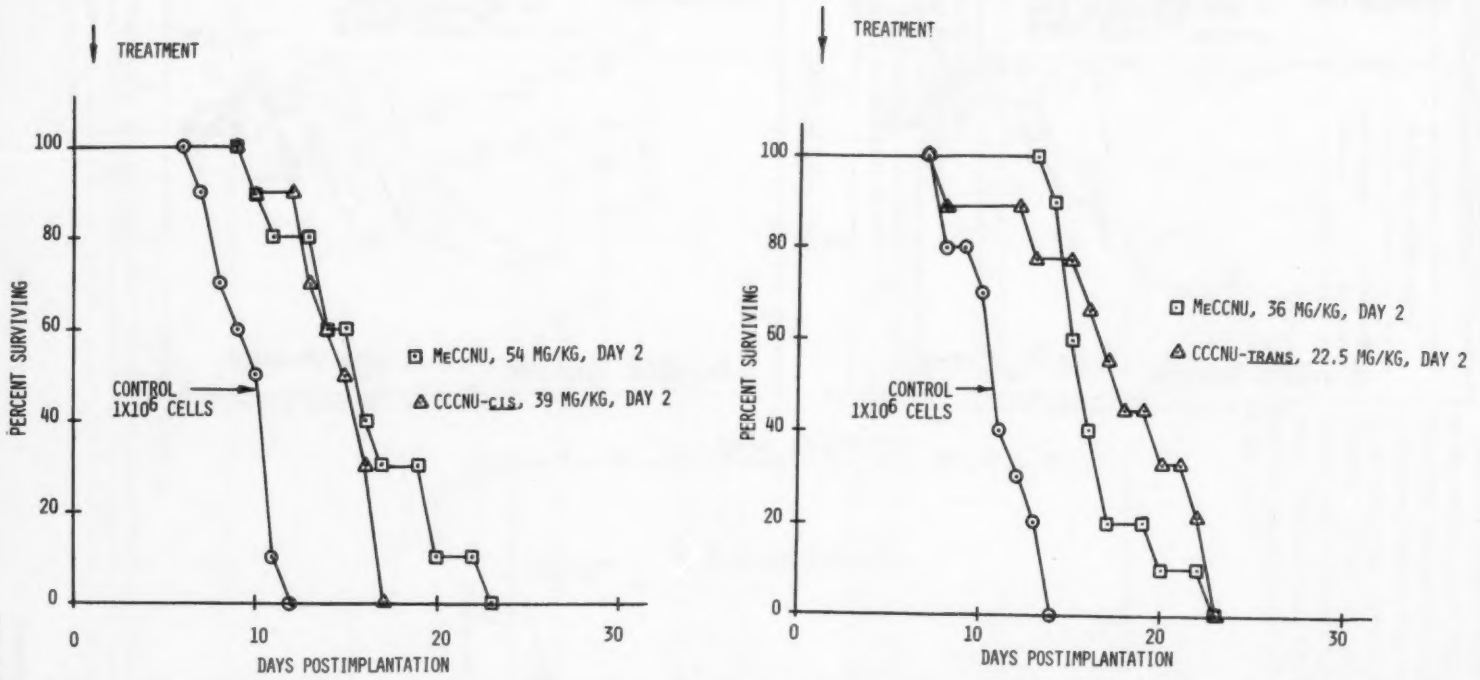


FIGURE 9.—Methyl-CCNU, CCCNU-*cis*, and CCCNU-*trans* treatment of mice implanted intracranially with B16 melanoma tumor cells. B16 tumor cells (1×10^6/BDF_1 mouse) were implanted intracranially on Day 0. The groups plotted are the highest nontoxic dose levels for each compound in these two experiments.

We found that a new water-soluble nitrosourea[7] was among the most active of the agents tested. The structure of this compound is shown in figure 6. The methyl group of methyl-CCNU was replaced by a carboxylic acid group. Both the *cis* and the *trans* forms of this compound have been synthesized and are equally active. The *cis* form of the drug is currently being used for most of the studies because of the greater ease of synthesis. The compound is soluble in a 1% pH 6.9 sodium phosphate buffer and is stable for 1 hour in this solution at temperatures of 4°C-10°C without loss of activity. CCCNU-*cis* has been found to be as active or more active than methyl-CCNU against six different transplantable tumors in mice. These tumors include: C3H mammary tumor, B16 melanoma, colon tumors, Lewis lung carcinoma,[8] P388 leukemia,[8] and L1210 leukemia.[8] An example of this activity against an intravenously (iv) implanted mammary tumor is shown in figure 7. Tumor cells (5×10^5) were implanted in all treatment groups on Day 0. Chemotherapy was carried out on Day 1. At optimum dose levels, methyl-CCNU cured three of ten animals, whereas CCCNU-*cis* cured eight of ten. A comparison of methyl-CCNU with CCCNU-*cis* against an advanced subcutaneously (sc) growing colon tumor is shown in figure 8. The water-soluble nitrosourea (CCCNU-*cis*) was again superior to methyl-CCNU. The water-soluble nitrosoureas are able to cross the blood-brain barrier although they are less lipophilic[9] than methyl-CCNU (*see* fig 9).

Advanced mouse colon tumors regressed under treatment with cyclophosphamide (fig 10). A schedule using high doses of cyclophosphamide spaced 1 week apart has been highly effective for this agent against several different transplantable solid tumors, including B16 melanoma, mammary tumors, and colon tumors.

In order for a colon-tumor model to simulate the human disease, it must respond well to the fluorinated pyrimidines. A schedule-dependency trial of 5-fluorouracil (5-FU) against trocar implants of one of the well-differentiated colon tumors is shown in figure 11. The four schedules attempted were highly effective. All of the colon tumors were also responsive to 5-fluoro-2'-deoxyuridine (5-FUDR). A daily-dose schedule (1-5 days or 1-7 days) was very effective for this agent (fig 12).

[7]Synthesized by T. Johnston and J. Montgomery; Southern Research Institute.

[8]Laster WR, and Schabel FM, Jr. Unpublished results.

[9]Johnston T. Water:octanol partition coefficient; log P value of methyl-CCNU = +3.3; log P value of CCCNU-*cis* at pH 7.4 = −1.1. Personal communication.

Surgical/Chemotherapeutic Adjuvant Studies

Two of the colon tumors appear to be usable for surgical/chemotherapeutic adjuvant studies. A preliminary experiment involving only surgical treatment of one of these tumors is shown in figure 13. Forty-five sc tumors were removed at three size ranges with a cutting cautery. Surgical failures (animal deaths with tumor regrowth at primary site) were excluded from this plot. As shown in figure 13, metastasis did not occur in a high percentage of the mice unless the tumors were over 600 mg at the time of surgery. Thus, surgical/chemotherapeutic adjuvant experiments were carried out only with these larger-sized tumors. An example of one such experiment is shown in figure 14. CCCNU-*cis* plus cyclophosphamide is a potentiating combination that produces a very high tumor-cell kill in the schedule and sequence shown. The results of the group receiving chemotherapy without surgical treatment are misleading in this experiment. Treatment was carried out on the same day as surgery (Day 15). At this time, the animals were severely debilitated from the effects of the tumor and could not tolerate even moderate doses of this combination. It is probable that smaller tumors would have regressed dramatically at these doses without lethality to the host.

Nitrosourea-Fluorinated Pyrimidine Combinations

There has been a great deal of interest in the nitrosourea-fluorinated pyrimidine combinations for the treatment of colorectal tumors (14). A surgical/chemotherapeutic adjuvant experiment with one of these combinations is shown in another paper in this issue (15). A 60% cure rate was obtained with surgical treatment plus methyl-CCNU and 5-FU as opposed to a 7% cure rate obtained with surgical treatment alone. The methyl-CCNU and 5-FU combination was also tested against far-advanced, well-differentiated colon tumors (table 6). The combination is at least modestly potentiating in this experiment.

The combination of methyl-CCNU and 5-FU has also been found to be modestly potentiating against advanced P388 leukemia[10] (table 7).

The use of another nitrosourea-fluorinated pyrimidine combination (BCNU and 5-FUDR) is shown in figure 15. In this case, the colon tumor cells were implanted iv and the treatment was delayed until Day 12 after implantation. At this time, the tumor growth in the lungs of the mice was moderately advanced and the animals had begun to lose weight from the effects of the tumor. The tumor cell kill was greater than additive for the BCNU-5-FUDR combi-

[10]Laster WR, and Schabel FM, Jr. Unpublished data.

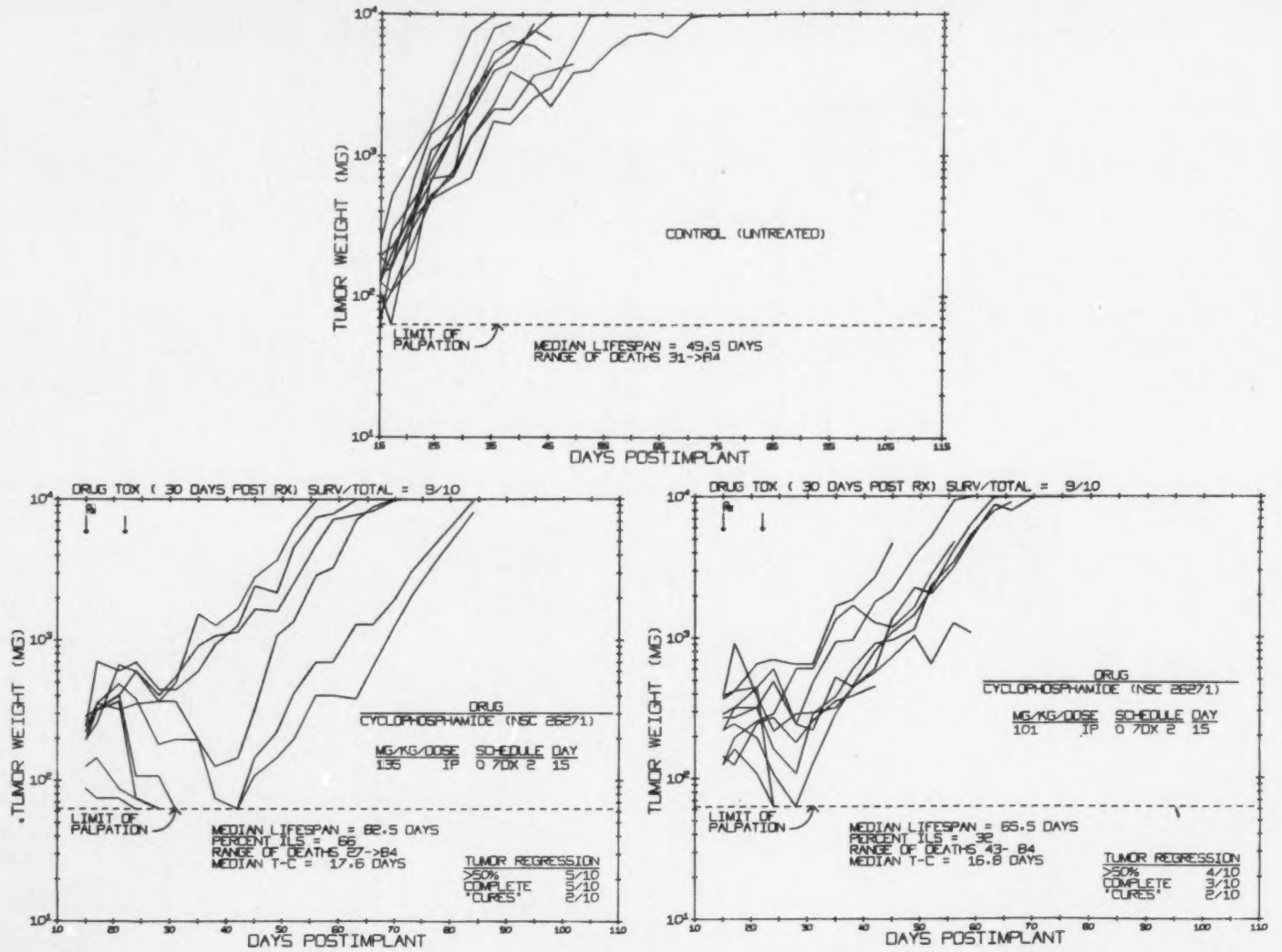


FIGURE 10.—Cyclophosphamide treatment of advanced colon tumors. Sc growing colon tumors (No. 36) were allowed to become large (100-400 mg) before treatment was initiated. Period of observation = 105 days.

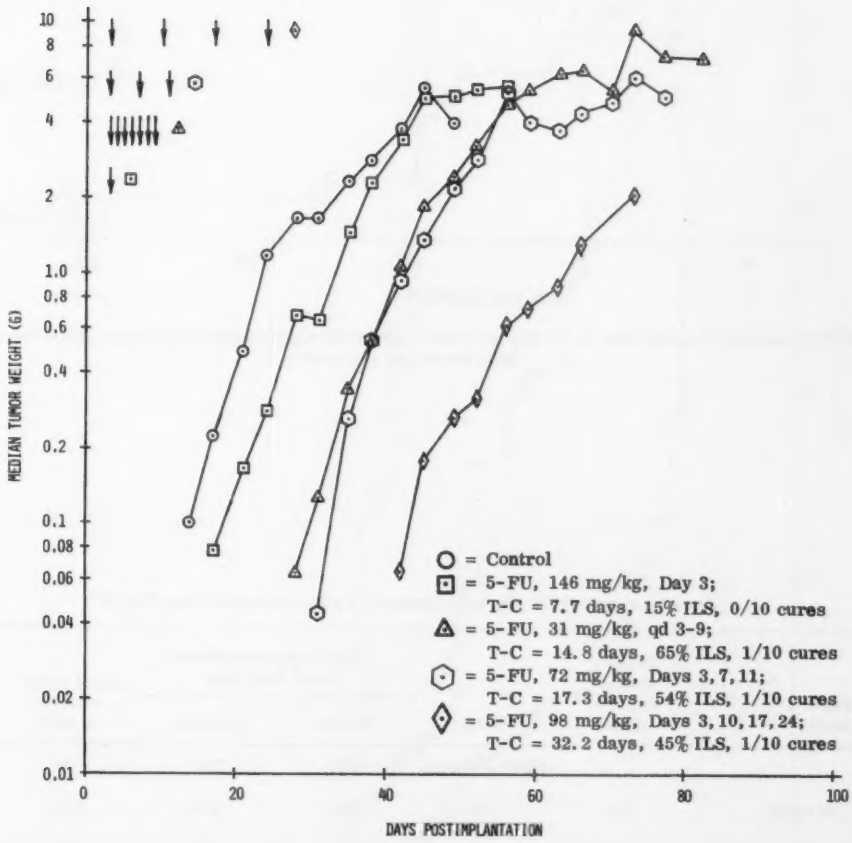


FIGURE 11.—Schedule-dependency trial of 5-FU: colon tumor No. 38. Trocar tumor fragments (20 mg each) were implanted into the axillary region of BDF_1 mice on Day 0. Chemotherapy with 5-FU was started on Day 3.

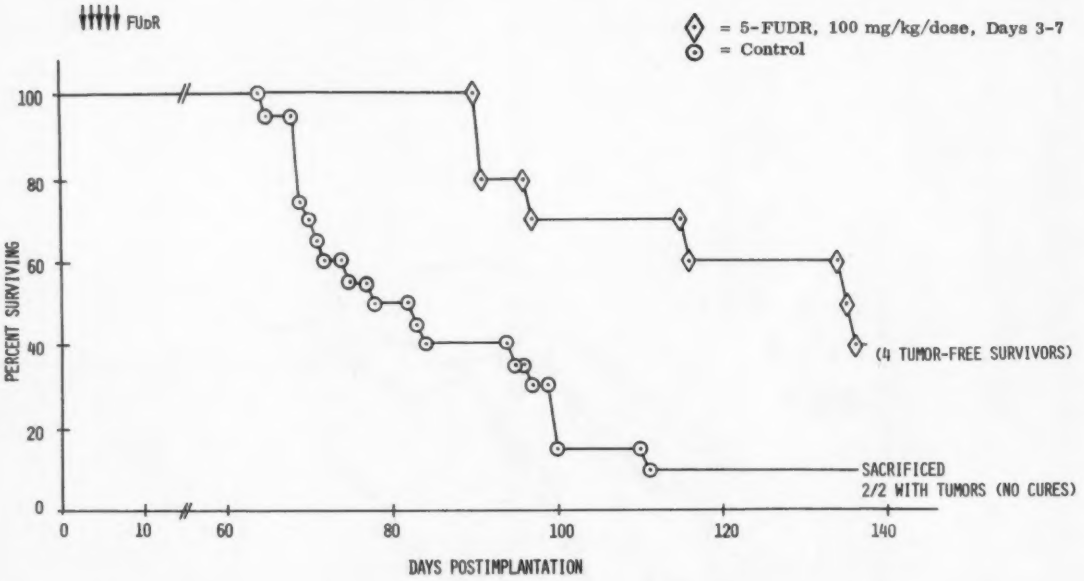


FIGURE 12.—5-FUDR treatment of colon tumor No. 38. Trocar tumor fragments (20 mg each) were implanted on Day 0. This was the 4th transplant generation of this tumor.

TABLE 6.—Methyl-CCNU plus 5-FU treatment of advanced colon tumors (No. 38)*

Optimum treatment—Days 14,21,28,35	Toxicity deaths/ total No. of mice	%ILS	No. of tumor regressions/ total No. of mice		No. of cures/ total No. of mice	No. of responders/ total No. of mice
			Partial	Complete		
Control		MDD† = 35 days	0/10	0/10	0/10	0/10
Methyl-CCNU, 20 mg/kg	3/10	+49	0/10	0/10	0/10	0/10
Methyl-CCNU, 13.3 mg/kg	0/10	+32	0/10	0/10	0/10	0/10
5-FU, 75 mg/kg	1/10	+64	1/10	0/10	0/10	1/10
Methyl-CCNU, 13 mg/kg + 5-FU, 50 mg/kg	0/10	+123	3/10	2/10	0/10	5/10

*Trocar tumor fragments (20 mg each) were implanted into the axillary region of BDF_1 mice. On Day 14 after implantation, animals bearing 100-400-mg tumors were randomized and allocated to the various treatment and control groups. Four weekly courses of chemotherapy were administered. Methyl-CCNU was injected ip 15-30 mins prior to the iv administration of 5-FU. Total responders represent both partial regressions (50% tumor reduction) and complete regressions (reduction below palpable size).

†MDD = median day of death.

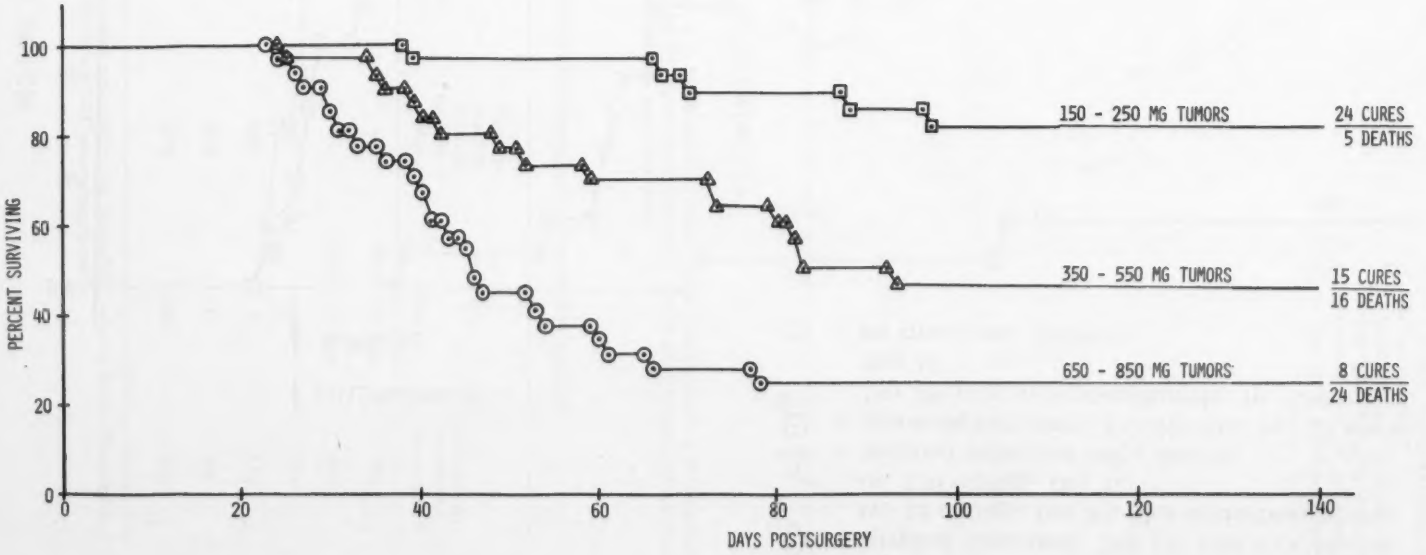


FIGURE 13.—Surgical removal of colon tumors: Balb/c mice died from metastases without primary site regrowth. Sc colon tumors (No. 26) were removed with a cutting cautery at three size ranges. There were 45 Balb/c mice/size range. Animal deaths with primary site regrowths (indicating surgical failure) were excluded.

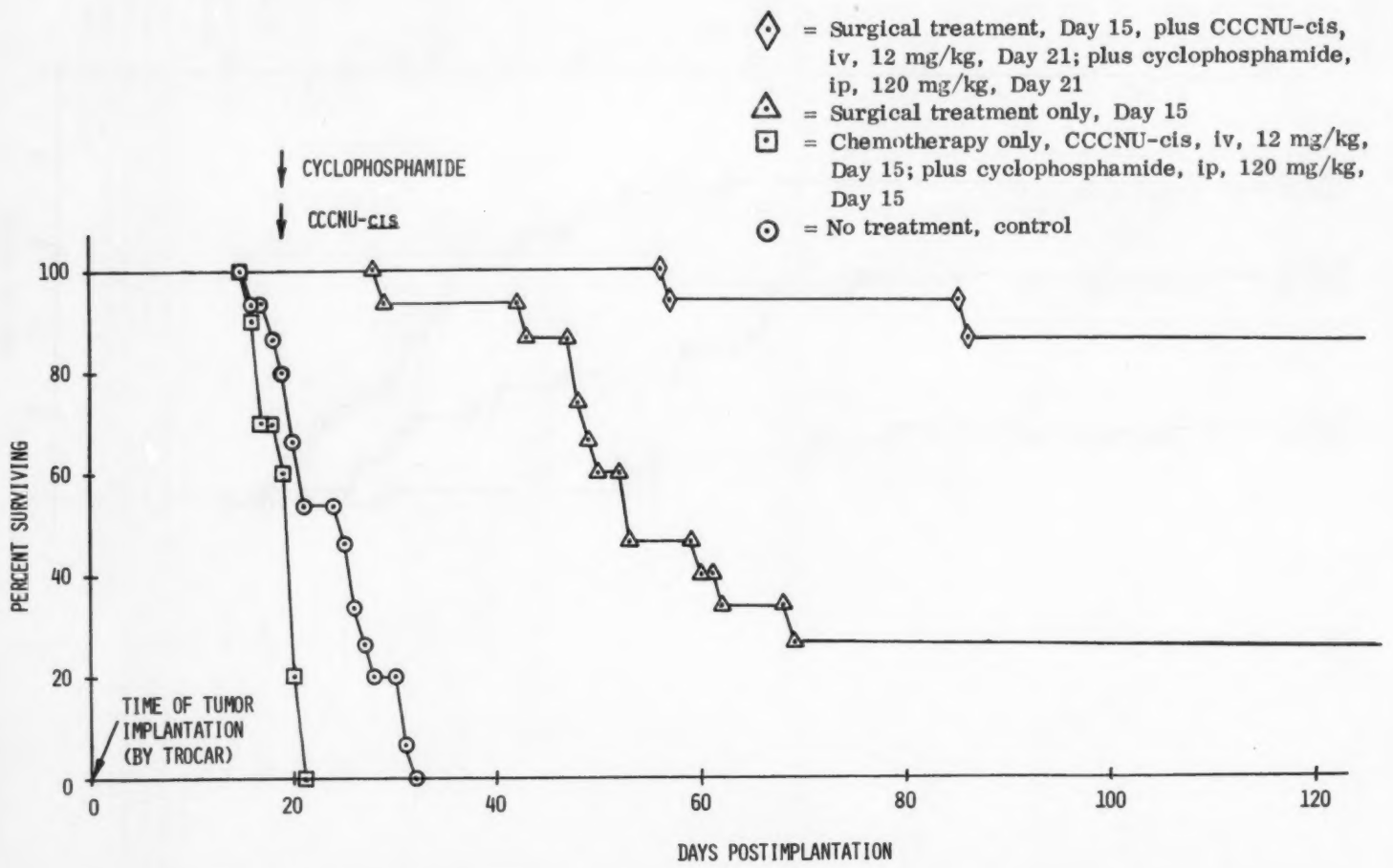


FIGURE 14.—Surgical treatment plus cyclophosphamide and CCCNU-*cis* for colon tumor No. 26. Sc growing colon tumors (600-1000 mg each) were removed with a cutting cautery on Day 15 after implantation. The animals were randomized immediately after surgery and were allocated to the surgical treatment only and surgical treatment/chemotherapy groups (15 animals/group). Six days later, cyclophosphamide was administered intraperitoneally (ip) at 120 mg/kg/dose. CCCNU-*cis* was given iv at 12 mg/kg/dose 15 minutes after the cyclophosphamide.

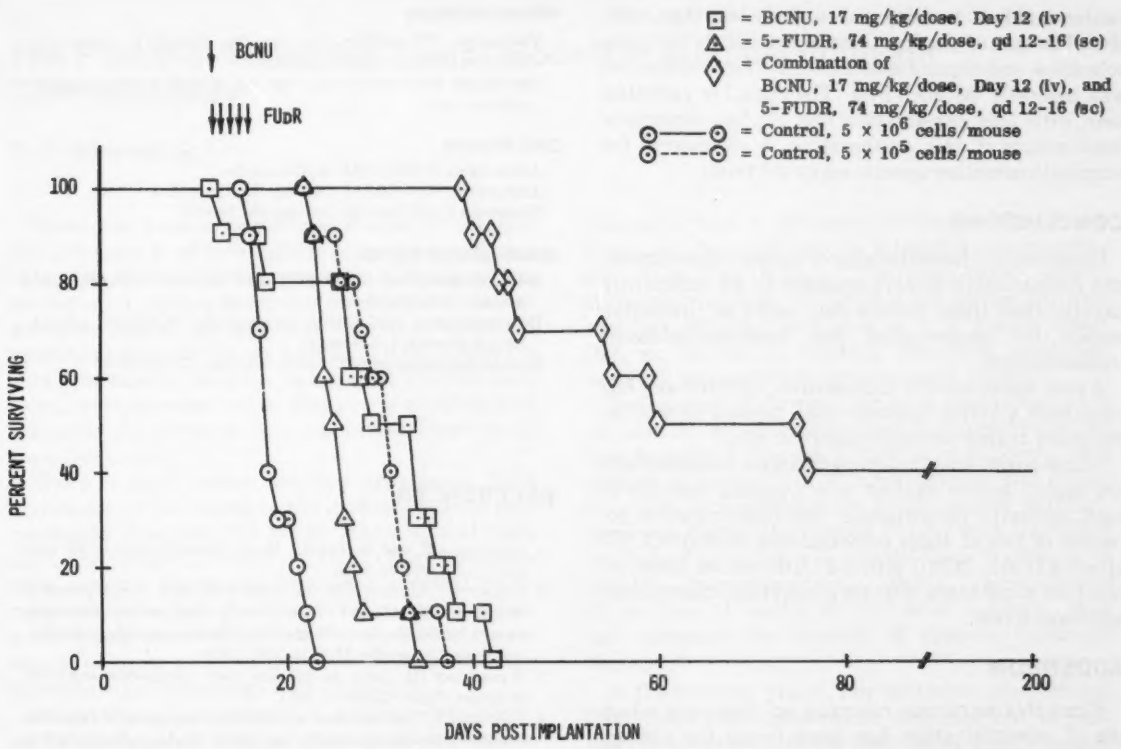


FIGURE 15.—BCNU-5-FUDR combination chemotherapy of iv implanted colon tumor No. 26. Titered cells (5×10^6 cells/Balb/c mouse) were implanted iv on Day 0. These mice were randomized and distributed among the various control and treatment groups. An additional group of ten mice was implanted with 5×10^5 cells to indicate the take rate of the tumor.

TABLE 7.—Methyl-CCNU plus 5-FU treatment of advanced P388 leukemia*

Optimum treatment—Days 6,10,14	Cells implanted on Day 0	Deaths in toxicity controls/ total No. of mice	%ILS	Survivors on Day 60/total No. of mice
Control	10^6	—	MDD† = 12 days	0/20
Control	10^5	—	MDD† = 17 days	0/10
Methyl-CCNU, 20 mg/kg	10^6	0/10	+158	1/10
5-FU, 80 mg/kg	10^6	0/10	0	0/10
5-FU, 60 mg/kg	10^6	0/10	+41	0/9
Methyl-CCNU, 15 mg/kg + 5-FU, 60 mg/kg	10^6	0/10	+233	4/10

*P388 leukemia cells (1×10^6/mouse) were implanted ip into female BDF_1 mice on Day 0. Chemotherapy was delayed until Day 6. Both methyl-CCNU and 5-FU were administered ip.

†MDD = median day of death.

nation and host toxicity was slightly less than additive. The sum of the fractions of the LD10s for these schedules and sequences is 1.25-1.3. The combination was modestly potentiating. The curative potential seen with this combination (fig 15) has important implications if this combination is considered for surgical/chemotherapeutic adjuvant trials.

CONCLUSIONS

Response to chemotherapy of mouse colon tumors and human colon tumors appears to be sufficiently parallel that these tumors may serve as predictive models for single-agent and combination-agent chemotherapy.

A new water-soluble nitrosourea, CCCNU-*cis*, has very high activity against colon tumors as well as five other transplantable tumors in mice.

Nitrosourea-fluorinated pyrimidine combinations are highly active against colon tumors and are at least modestly potentiating. The high curative potential of two of these combinations (methyl-CCNU plus 5-FU and BCNU plus 5-FUDR) makes them attractive candidates for surgical/chemotherapeutic adjuvant trials.

ADDENDUM

Since this work was reported, an improved schedule of administration has been found for methyl-CCNU plus 5-FU: the administration of both agents on the same day (methyl-CCNU given 60-90 minutes prior to 5-FU) at 13-day intervals. This longer interval between courses produced results superior to those shown in table 6.

LIST OF COMPOUNDS EVALUATED

Alkylating Agents

Ara-C: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride; cytosine arabinoside

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

CCCNU-*cis*: NSC-153174; CAS reg. No. 42558-93-0; cyclohexanecarboxylic acid, 4-[3-(2-chloroethyl)-3-nitrosoureido]-, *cis*-

CCCNU-*trans*: NSC-153175; CAS reg. No. 42558-94-1; cyclohexanecarboxylic acid, 4-[3-(2-chloroethyl)-3-nitrosoureido]-, *trans*-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-; MeCCNU

Antimetabolites

5-Fluoro-2'-deoxyuridine: NSC-27640; 5-FUDR

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Methotrexate (Amethopterin): NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[p-[[(2,4-diamino-6-pteridinyl)methyl] methylamino]benzoyl]-

PalmO-ara-C: NSC-135962; CAS reg. No. 31088-06-9; cytosine, 1-β-D-arabinofuranosyl-, 5'-palmitate

Mitotic Inhibitors

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

DNA Binders

Actinomycin D: NSC-3053; dactinomycin

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8

Mitomycin C: NSC-26980; CAS reg. No. 50-07-7

Miscellaneous Agents

Dianhydrogalactitol: NSC-132313; CAS reg. No. 23261-20-3; galactitol, 1,2:5,6-dianhydro-

Dibromodulcitol: NSC-104800; CAS reg. No. 10318-26-0; galactitol, 1,6-dibromo-1, 6-dideoxy-

Hexamethylmelamine: NSC-13875; CAS reg. No. 645-05-6

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The Potential for Murine Tumor Models in Surgical Adjuvant Chemotherapy[1]

D. P. Griswold, Jr.[2,3,4]

There has been a marked escalation in interest and attempts to obtain significantly increased cure rates among the solid cancers in man. Senator Norris Cotton (1), now a member of the National Cancer Advisory Board, perhaps best expressed this view when he remarked: "In two months, I'll be 75, and I'm very interested if someone tells me I'll live five more years, or three more years. But young people aren't impressed by five-year survival rates. They live in hope...[of]...cure."

There is good reason for this impatience. Take breast cancer for example, the leading cause of cancer deaths in women. For the 10-year period of 1955-1964, the relative 10-year survival rate of patients with regional disease who received surgical treatment only was just 43% (2). Yet during the subsequent 5-year period of 1965-1969, only 10% of breast cancer patients received any form of chemotherapy in conjunction with surgical treatment (3).

Reluctance of the surgeon to employ chemotherapy in conjunction with or shortly after surgical treatment was based more on reality than hope. When the surgeon viewed chemotherapeutically induced tumor-response rates of 35%-40% at best, and being cognizant that those responses were more often than not partial and transient in nature, having little, if any, effect on survival time, it is understandable that consideration of chemotherapy flew in the face of his ethic to do his patient no harm. In considering reasons for chemotherapeutic failure, Carter (4), however, has very appropriately pointed out that "an additional factor, which is often neglected, is the point in the treatment strategy for a given disease at which chemotherapy is introduced. In this respect, it is significant that the local and regional modalities of surgery and radiotherapy are commonly used in primary treatment of solid tumors, and chemotherapy is relegated to secondary use after their failure." Unquestionably, single-modality treatment of operable but metastatic cancers has definite limitations as it is known today. But, as DeVita (1) recently remarked, combined-modality therapy offers much greater promise.

The concept of surgical adjuvant chemotherapy is not new. As attested to by literature reports, it has been supported by a small nucleus of believers for a number of years (5-15). It is noteworthy that Fisher et al (16), in describing the breast cancer surgical-adjuvant studies that began in 1957 with thioTEPA and 5-fluorouracil (5-FU), stated that: "failure ... of the two chemotherapeutic agents ... administered at the time of and/or shortly after surgery...does not repudiate the concept of systemic adjuvant therapy."

In the ensuing years, few well-controlled clinical trials have been undertaken. Standing out as hallmarks, however, are: the melphalan breast cancer study reported by Fisher et al (17), the adriamycin osteosarcoma study reported by Cortes et al (18), and the methotrexate osteogenic sarcoma study reported by Jaffe et al (19). The two most recent studies (17,18) have now demonstrated the effectiveness of chemotherapy as an adjuvant to surgical treatment in a clinical setting. The concept, based principally on experimental evidence until this time, has now become principle, translatable from animal to man. As noted by DeVita last fall, it requires no further proof.[5]

With evidence of a growing interest and need for surgical adjuvant chemotherapy, it has been our intent to determine, quantitatively, optimal approaches to surgery/chemotherapy, using existing models where applicable and developing new models where appropriate ones were unavailable. Four years ago, Skipper (20) reviewed some basic chemotherapeutic concepts, with emphasis on disease staging and surgical adjuvant chemotherapy, considering the relevancy of experimental results to possible clinical application. In the present paper I would like to extend those views in light of more recent knowledge, looking at factors that appear to be

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[5]DeVita VT. Concluding remarks at the Division of Cancer Treatment contractors meeting, Lancaster, Pa, 1974.

helpful, if not essential in the design of surgical/chemotherapeutic trials. In this context, I would like to consider (*a*) several factors that appear to be relevant in the validation of our so-called models and (*b*) factors that bear on the design of surgical/chemotherapeutic trials. These factors include: (*a*) growth, metastatic, and response characteristics that affect the authenticity of the model systems; (*b*) the suitability or appropriateness of measurement endpoints; (*c*) the effect of tumor stage on "curability"; and (*d*) some essential factors to the development of optimal therapeutic regimens.

MATERIALS

The animal tumors referred to in this paper are: (*a*) B16 melanoma in the BDF_1 ($C57BL/6 \times DBA_2$) mouse (21); (*b*) mammary carcinoma in the C3H/HeN mouse; (*c*) Lewis lung carcinoma in the BDF_1 mouse (14); (*d*) colon carcinoma in the BALB/c mouse (22); and (*e*) plasmacytoma in the hamster (23).

The chemotherapeutic agents discussed are methyl-CCNU, cyclophosphamide, 5-FU, and CCCNU-*cis*.

RESULTS AND DISCUSSION

Validity of the Model

It is incumbent upon the experimentalist to show that his animal tumor system is not just another tumor but is indeed a model for some human cancer. Biologic characterization of the animal tumor should show certain similarities to specific characteristics of the human cancer. In the broadest terms, this might include such characteristics as growth behavior, tumor lethality, metastasis, and chemotherapeutic predictiveness.

Previously Dr. Skipper reported a method for determining the growth rate of a solid tumor from a single cell through the visible range of growth (20). Our total experience with B16 melanoma is shown in figure 1. Construction of this tumor-growth curve was based on calculation of the time (in days) required for specific numbers of cells to reach a prede-

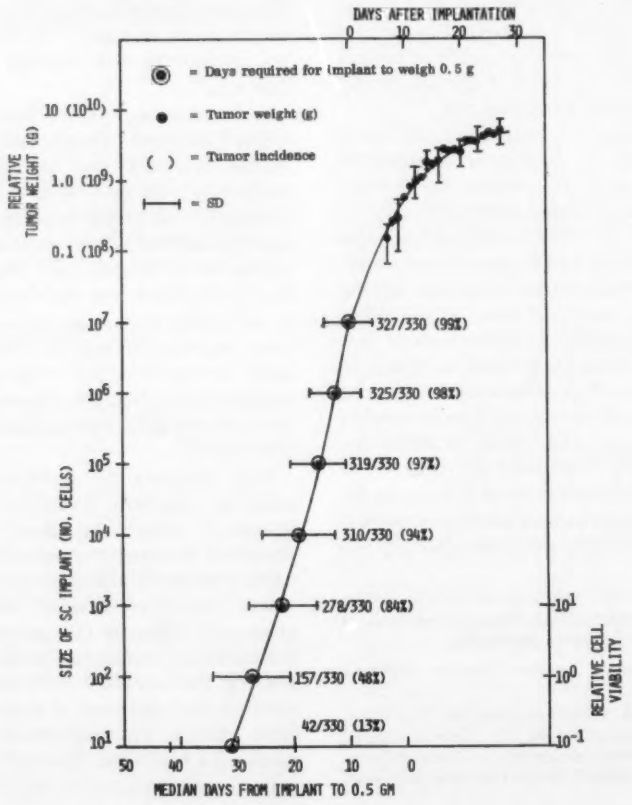

FIGURE 1.—Growth of sc implanted B16 melanoma after implantation of 10^7 cells and $\log_{10}$ dilutions. A 20% brei of lethally irradiated (10,000 rads cobalt 60) cells (about 5×10^7 cells per implant) was used as the diluent. Curves were visually constructed.

termined measurable size along with actual measurements of those tumors as they became palpable. Application of the Poisson distribution function to the tumor-incidence rates for the various cell titrations allowed estimation of relative viability. In this instance it was estimated to be about 1.0%.

In a similar fashion, growth curves were constructed for the Lewis lung carcinoma and the C3H mammary carcinoma, shown with the growth curve of the B16 melanoma as a composite plot in figure 2. Although the growth of each tumor became distinctly asymptotic and at different rates, it is interesting that during the first few decades of growth the differences in growth rates were minimal. Estimation of the overall (ie, from 1.0 cell to 1.0 g) average volume doubling times showed a twofold variation with rates of 1.0-2.0 days. Knowledge of tumor-growth rates during early growth stages is essential in surgical adjuvant situations where the experimentalist may be dealing with tumor masses that are not visible. Estimation of overall average volume doubling times from measurements made during the asymptotic period of tumor growth may be quite misleading; furthermore, when one considers that the mouse ages approximately 30 times faster than man, tumor volume doubling times of 1.0-2.0 days, early in the life of the tumor, are not unreasonable. The absurdity of a requirement of overall average volume doubling times of, say, 30 days is made clear by calculations which show that if a mouse's tumor of 1.0 or even 100 cells was induced at birth and doubled every 30 days, the mouse would be dead before his tumor was clinically detectable.

Another point the experimentalist must consider is the lethality and manner of lethality of his animal tumor. For surgical adjuvant studies, tumors that spontaneously regress and fail to kill or that kill the host by some unknown manner (sometimes described as a humoral factor) are not suitable. Based on data of Cutler and Myers (24) (table 1), one can see a definite relationship between tumor size and time of patient death. Comparisons of tumor implant size and time of host death have been made with a variety of animal tumors (20). The comparison is shown in table 2 for B16 melanoma and in tables 3 and 4 for two different lines of C3H mammary carcinoma.

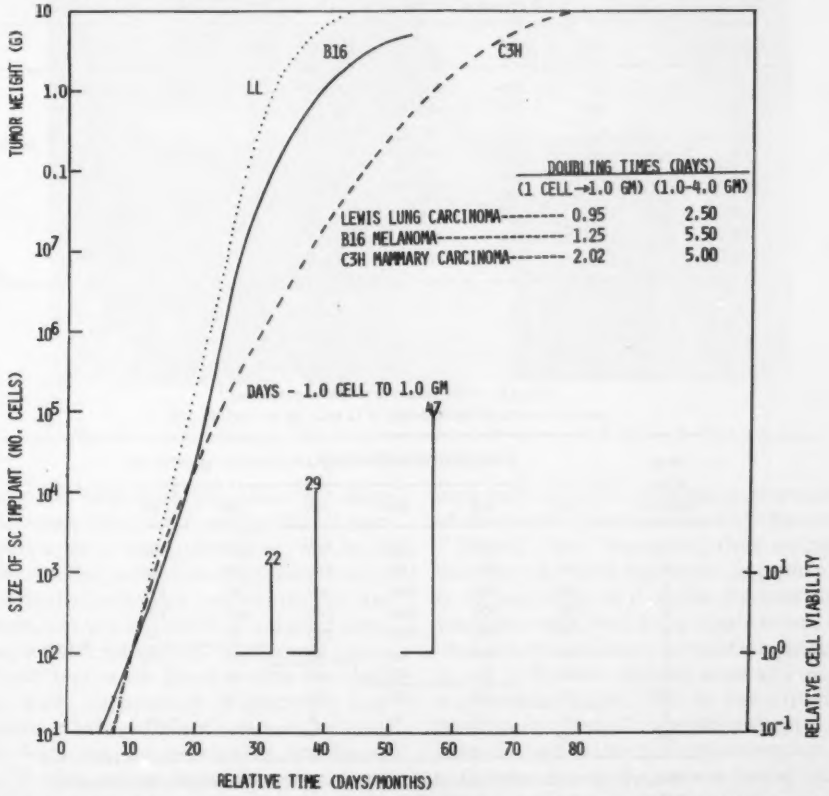

FIGURE 2.—Growth of sc implanted Lewis lung carcinoma (LL), B16 melanoma, and C3H carcinoma after implantation of 10^7 cells and $\log_{10}$ dilutions. See fig 1 legend.

TABLE 1.—Breast cancer in women: percent alive, of those dying in 10 yrs, by tumor size at time of surgery*

Yrs after diagnosis	Percent alive with tumor size (cm) at time of surgery of—								
	<1.0	1-2	2-3	3-4	4-5	5-7.5	7.5-10	10	
1	100	89	95	91	92	89	71	81	(50%)
2	76	79	80	71	75	67	50	43	
3	57	64	67	53	56	48	21	26	
4	50	57	50	40	40	37	15	7	
5	50	43	38	28	33	27	7	7	
6	38	32	29	20	23	17	4	0	
7	19	20	20	14	16	12	4		
8	19	18	14	9	9	11	0		
9	0	5	7	3	4	5			

*From ref 24.

TABLE 2.—B16 melanoma in the mouse: percent alive, of those dying in 10 wks, by sc implant size

Wks after implant	Percent alive with sc implant size (No. of cells) of—							
	10^1	10^2	10^3	10^4	10^5	10^6	10^7	
1	100	100	100	100	100	100	100	
2	100	100	100	100	100	89	90	
3	100	100	100	100	100	78	70	
4	100	100	100	90	80	33	30	(50%)
5	100	83	50	60	10	12	10	
6	50	50	30	10	10	0	0	
7	50	33	10	0	0			
8	0	33	0					
9	0	0						

TABLE 3.—C3H mammary adenocarcinoma:* percent alive, of those dying in 10 wks, by sc implant size

Wks after implant	Percent alive with sc implant size (No. of cells) of—						
	10^2	10^3	10^4	10^5	10^6	10^7	
1	100	100	100	100	100	100	
2	100	100	100	100	100	100	
3	100	100	100	100	100	100	
4	100	100	100	100	100	100	
5	100	100	100	100	50	60	
6	100	100	80	60	30	30	(50%)
7	100	80	60	30	10	0	
8	50	40	20	10	0		
9	25	0	0	0			
10	0						

*Mouse tumor cells were implanted sc using a 20% brei of lethally irradiated (cobalt 60) tumor cells (about 5×10^7 cells/implant) as the diluent.

TABLE 4.—C3H mammary carcinoma:* percent alive, of those dying in 10 wks, by sc implant size

Wks after implant	Percent alive with sc implant size (No. of cells) of—						
	10^1	10^2	10^3	10^4	10^5	10^6	
1	100	100	100	100	100	100	
2	100	100	100	100	100	100	
3	100	100	100	100	100	90	
4	100	100	100	89	100	10	(50%)
5	100	75	55	44	22	0	
6	50	25	11	0	0		
7	25	25	0				
8	25	12					
9	0	0					
10							

*Mouse tumor cells were implanted sc using a 20% brei of lethally irradiated (cobalt 60) tumor cells (about 5×10^7 cells/implant) as the diluent.

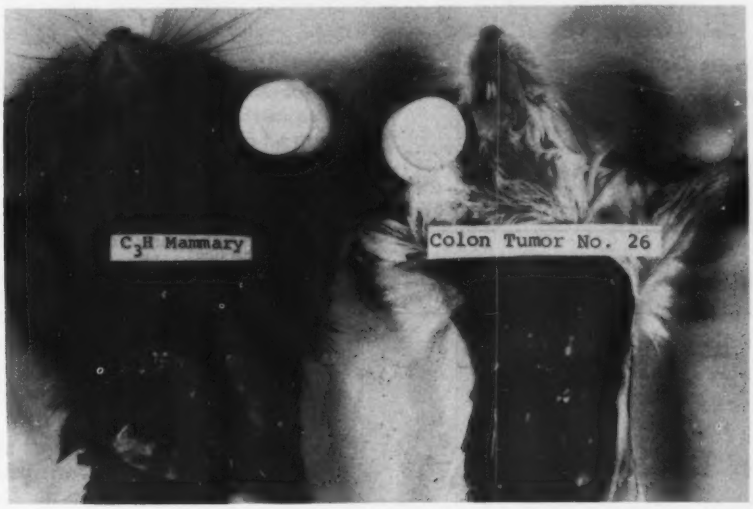

FIGURE 3.—Thoracic cavity metastasis after surgical removal of sc implanted tumors at 0.5-1.0 g. (Liver and diaphragm removed.)

We have, however, been unable to observe a clear relationship between host death and growth of metastatic foci from some of our tumor lines. Wexler et al (25) have shown that a number of animal tumors do indeed metastasize, although metastatic foci are only microscopic. For use as potential surgical adjuvant tools, we are not interested in animal tumors whose metastatic foci never become more than microscopic. For such purposes it is essential that metastatic lesions attain sufficient size and distribution that host death may be reasonably attributed to metastasis. To that end we have chosen or developed (primarily by selective transplantation) specific tumor lines (of breast, lung, colon, and melanoma) that have suitable metastatic characteristics. Four of these tumor lines are shown in figures 3 and 4.

Finally, it is important that animal tumors respond to the same agents as do human tumors. This is not as simple as it seems, because the implication has been made that for a *single* mouse tumor to be a chemotherapeutically predictive model it must respond in the same manner as do all human tumors (of a particular type). But in our experience, heterogeneity, in terms of chemotherapeutic response, has been the rule rather than the exception.

In table 5 it is shown how ten of our C3H breast tumor lines responded or failed to respond to a selected list of clinically effective agents. Each line

FIGURE 4.—Thoracic cavity metastasis after surgical removal of sc implanted tumors at 0.5-1.0 g. (Liver and diaphragm removed.)

TABLE 5.—Relative response of various transplantable lines of C3H mammary tumors [data of T. H. Corbett]

Drug	Tumor line*									
	44	10A	16C	11C	13C	10C	15C	04A	07A	08A
Cyclophosphamide	3	3	3	3	4	2	3	3	3	3
Melphalan	1	2	2		1		—	3	2	2
Adriamycin	1	3	3	3	4	3	2	3	2	
5-FUDR	3	1		1		2	2	—	1	1
5-FU	—	2	2	—	1		3	3	2	
Vincristine	—	—	2	—	2		—	1	1	
Methotrexate	—	—	—	—	—	—	—	—	—	—
Prednisone	—	—	1		1		—	3	—	

*— = No activity; 1 = marginal activity; 4 = some "cures" after early treatment of trocar implants; no symbol = not tested.

was derived from a single and distinct spontaneous tumor. Variation in response was considerable. None of our lines have been remarkably responsive to methotrexate and relatively few to vincristine and prednisone. Sixteen different treatment schedules were used for the evaluation of methotrexate. A number of different schedules were used for the evaluation of the other agents, but they were by no means all-inclusive. Nevertheless. heterogeneity in terms of chemotherapeutic responsiveness of the different tumor lines is obvious. For example, adriamycin, which is markedly effective against line 13C, was only marginally active against line 44. Where line 44 responded well to 5-fluorodeoxyuridine (5-FUDR), line 04A failed to respond to treatment with that agent. It may be possible that human tumors show this same variation in response, even tumors within a particular morphologic type. This is important to consider, particularly when comparing the percent of responders in combination-agent trials, whether experimental or clinical. An increased percentage of responders to multiple-agent therapy, when dealing with spontaneous or induced tumors, may reflect heterogeneity rather than true potentiation.

In any event, knowledge of the chemotherapeutic responsiveness of particular tumor lines and sublines together with knowledge of tumor growth and metastatic characteristics provides a basis on which to judge whether an animal tumor may be considered

a "model." And it seems imperative that tumor responsiveness be based on the most meaningful, as well as practical, endpoints.

Suitability of Endpoints

The commonly used measurement endpoints for assessing the antitumor activity of a drug (or irradiation or immunotherapy) include the percent increase in host lifespan (%ILS), the percent of control tumor weight inhibition (%T/C), and the delay in days of visible tumor appearance or recurrence (T-C). The experimentally used %ILS and %T-C values correlate with the clinically used terms, survival time and duration of remission. The %T/C value has no commonly used clinical correlate; percent of responders, a count of the number of tumors or patients with tumors that regress by 50% or more, only recently has been used to any major extent experimentally (26). In the absence of suitable biochemical markers that might be used as measures of the number of tumor cells present before and after therapy, such intermediate endpoints as described above have been useful in determining the effectiveness of various therapies. Their use, however, can be quite misleading. Ideally, one should know the percent of cells killed or the number of viable cells remaining after a dose or course of treatment.

While it seems clear that a count of the number of responders[6] in a treated group is not a measure of the cell kill obtained, if any, it is equally important to recognize that failure of an advanced tumor to respond (ie, regress by 50% or more) may not necessarily reflect a drug's antitumor inactivity. Comparison of the percent of responders among advanced tumors with assayed cell kill from companion groups, for two different types of tumors, after treatment with methyl-CCNU or cyclophosphamide showed a lack of correlation between the two endpoints (table 6). Where a cell kill of about 3.5 $\log_{10}$ units was measured in B16 melanoma after treatment, only 6% of the companion tumors regressed by 50% or more; but 96% of hamster plasmacytomas regressed while assay indicated only about 2 $\log_{10}$ units of cell kill. The lack of correlation may be due to a combination of growth characteristics, including proliferative rate, growth fraction, rate of lysis and resorption, and normal cell loss (27-29).

Measurements of host survival time or delay in tumor recurrence are more meaningful endpoints in that they provide a means, albeit crude, of estimating the reduction of cell viability after a dose or course of treatment. But results from these endpoints, too, may be misleading, particularly if the schedule or regimen of treatment is ignored.

In idealization (fig 5) of the response of B16 melanoma to three different treatment schedules of cyclophosphamide (single dose, every 4 days × 4, and daily for 9 days), one can see that the delay in median tumor appearance to a predetermined size was about the same (15-19 days) regardless of the treatment schedule employed. Similarly, there was no significant difference in ILS among the three groups. The differences in net cell kill, however, were remarkable. We may conclude that the possibility of tumor cell proliferation between doses or courses of treatment must be taken into account. The host recovery times shown here are purely arbitrary, allowing about 2 weeks from the end of each of the three

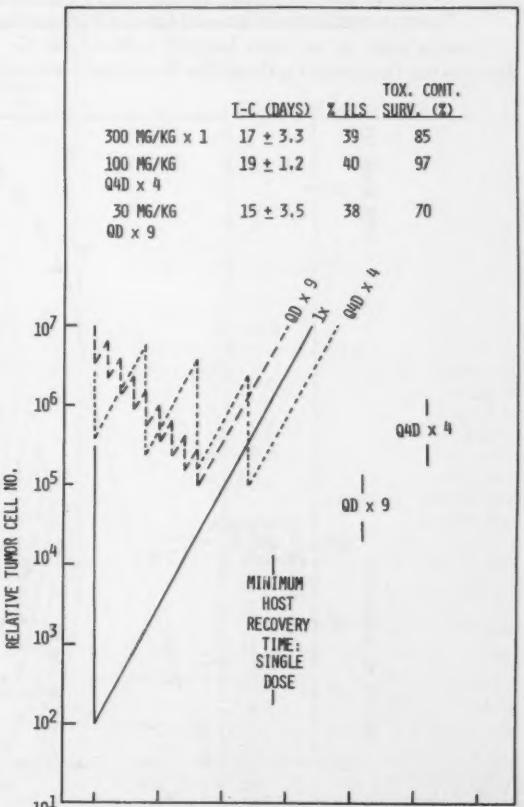

FIGURE 5.—Idealized response of sc implanted B16 melanoma to cyclophosphamide when used in three different treatment regimens. Based on three or more experiments per treatment schedule. Host recovery times arbitrarily set at 14 days after termination of treatment.

[6]Responder is used here in the classic sense to indicate regression of tumor volume or mass ≥ 50%.

TABLE 6.—Comparison of percent responders to tumor cell kill

Experiment No.	Agent	No. of responders/ total No. treated*	Percent responders	Assayed cell kill (log_{10} units)
B16 melanoma				
1021-G_1	Methyl-CCNU	0/10	0	5
1202-A_1	Methyl-CCNU	0/10	0	4
1048-D_2	Methyl-CCNU	1/20	5	3
1200-A_3	Methyl-CCNU	1/10	10	4
1399-C_1	Cyclophosphamide	2/4	50	3
1202-B_1	Cyclophosphamide	0/10	0	3
Total=		4/64	6	about 3.5
Plasmacytoma No. 1				
CK-135	Cyclophosphamide	10/10	100	2
CK-204	Cyclophosphamide	19/20	95	3
CK-246	Cyclophosphamide	19/20	95	2
Totals		48/50	96	about 2

*Responder = observed regression of tumor mass $\geq$ 50%.

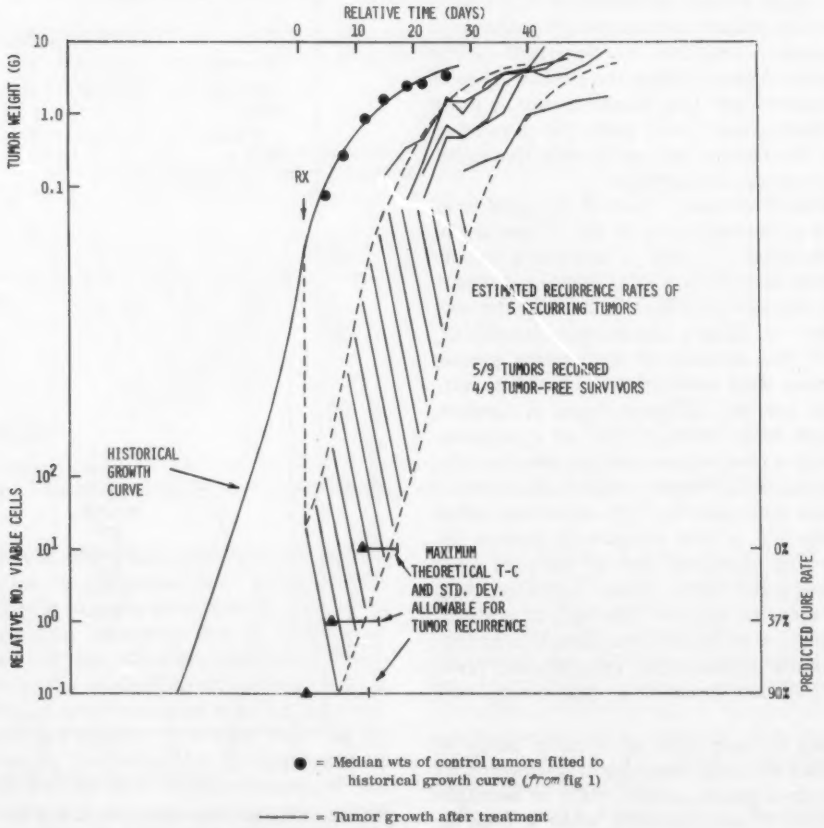

FIGURE 6.—Response of sc implanted B16 melanoma to methyl-CCNU, 54 mg/kg on Day 2 only, and idealized recurrence rates of tumors not "cured."

treatments. They were included only to show their relative relationship, pointing out the greater likelihood of failure for the multiple-dose regimens. Had this been a surgical adjuvant trial, where 10^3 or 10^4 tumor cells were the target, the choice of treatment schedules would be obvious.

These data should by no means be taken to imply that a single dose of an agent is always optimal. Many factors affect drug scheduling, including tumor, host, and drug characteristics. The data do suggest the need for the determination of the reduction of tumor-cell population viability. That is, whatever endpoints are used must be converted to cell kill or to surviving fraction. That statement, of course, raises the following question: Can reasonable estimates of cell kill be made from an intermediate endpoint such as duration of remission or delayed tumor recurrence?

In figure 6, we have reproduced the historical overall growth curve of B16 melanoma (*from* fig 1) and we tacitly assumed it to reflect the repopulation rate of tumors during recurrence after treatment. Control tumor weight data points from another experiment were fitted to that curve to determine the likely adequacy of that curve to describe growth of untreated tumors in this single experiment. The goodness of fit is apparent. The unbroken lines represent measured growth of the five of nine tumors that recurred after treatment with a single dose of methyl-CCNU (about 1.35 LD10). Extrapolations of recurrence rates were based on the slope of the historical growth curve. In this experiment, four of nine mice were "cured" of their tumors; thus, the relationship between tumor growth delay (T-C) and reduction in cell population viability was, in this single instance, authenticated by "cure" in association with the delay theoretically predetermined (about 20-25 days) to equate with "some cure."

Crude as this method may be, it does appear to provide a means of estimating fractional survival of

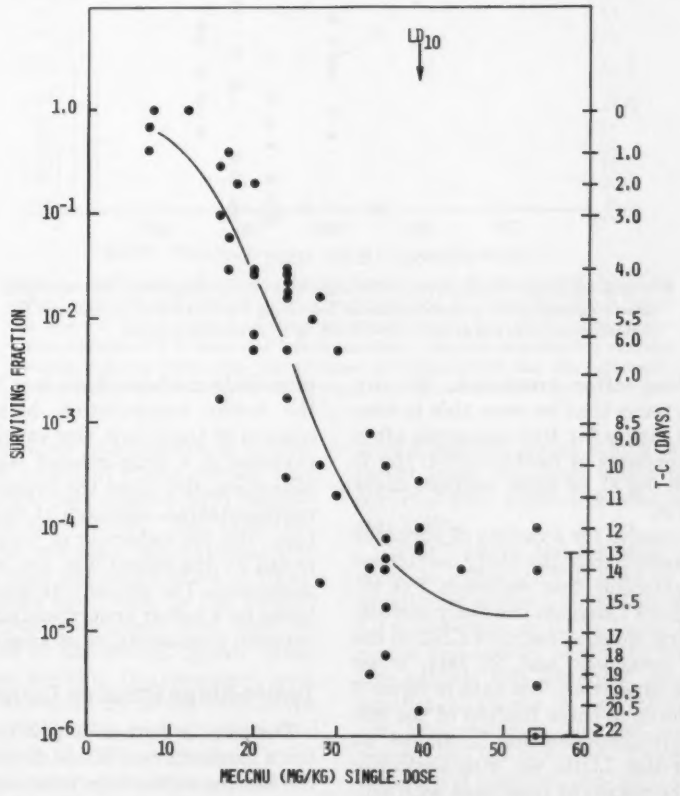

FIGURE 7.—Estimate of cell survival of sc implanted (trocar fragments) B16 melanoma after treatment with methyl-CCNU. Surviving fraction is based on observed T-C in relation to historical growth curve (*from* fig 1); 10 mice/data point.

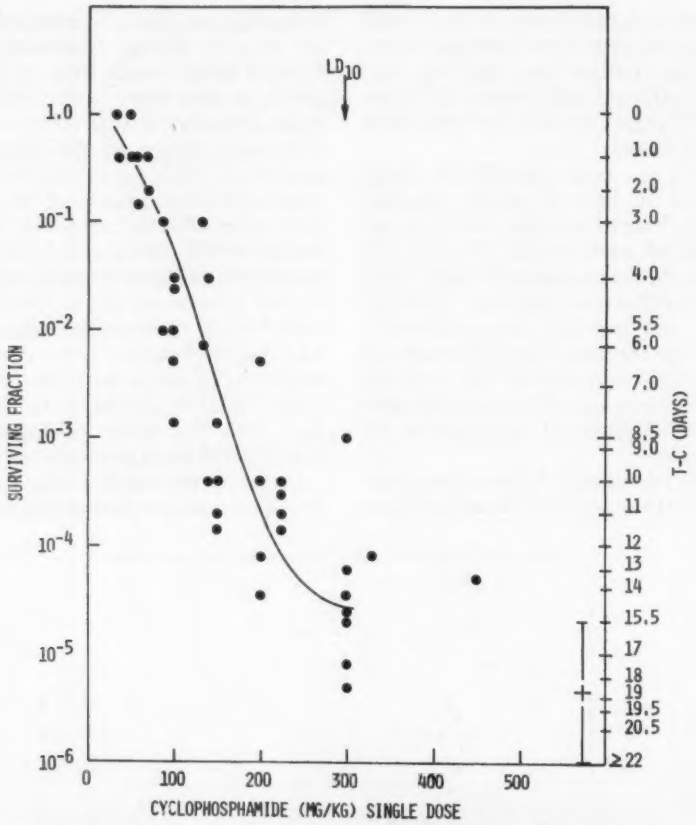

FIGURE 8.—Estimate of cell survival of sc implanted (trocar fragments) B16 melanoma after treatment with cyclophosphamide. Surviving fraction based on observed T-C in relation to historical growth curve (*from* fig 1); 10 mice/data point.

tumor-cell populations after treatment. In any event, it was on this basis that we were able to construct dose-survival curves for B16 melanoma after treatment with single doses of methyl-CCNU (fig 7) or cyclophosphamide (fig 8), or after multiple doses of methyl-CCNU (fig 9).

These curves are useful for a variety of purposes and may allow the prediction of the likely usefulness or the futility of particular dose regimens. For example, the data in figure 7 suggest the likely uselessness of low continuing doses of methyl-CCNU in the treatment of B16 melanoma and, in fact, other results[7] confirm that prediction. The data in figure 8 indicate that two thirds to three fourths of the single-dose LD10 of cyclophosphamide is almost as effective as the full-dose LD10. yet with obviously less host toxicity. The results of treatment with multiple-dose schedules (fig 9) present a problem in interpretation where there is a lack of knowledge of the tumor repopulation between treatments or courses of treatment. Our experience suggests little evidence of a drug-induced lag after treating B16 melanoma, therefore the broken line would be most representative, although at this point it is not certain. The net reduction of viable cells would be depicted by the dotted line, too wide a range to be of usefulness. The proper interpretation of these data, based on a better understanding of the repopulation between treatments, must await further study.

Tumor-Stage Effect on Curability

The data in figures 10 and 11 show that "cure" is not a random event but is directly related to tumor-cell burden at the time treatment is initiated. When counted numbers (10^7, 10^5, and 10^3) of B16 melanoma cells were implanted sc, all untreated mice died as a result of tumor growth (fig 10). When companion groups of mice (bearing 10^7, 10^5, or 10^3 cells) were

[7]Dykes DJ, Southern Research Institute. Unpublished data.

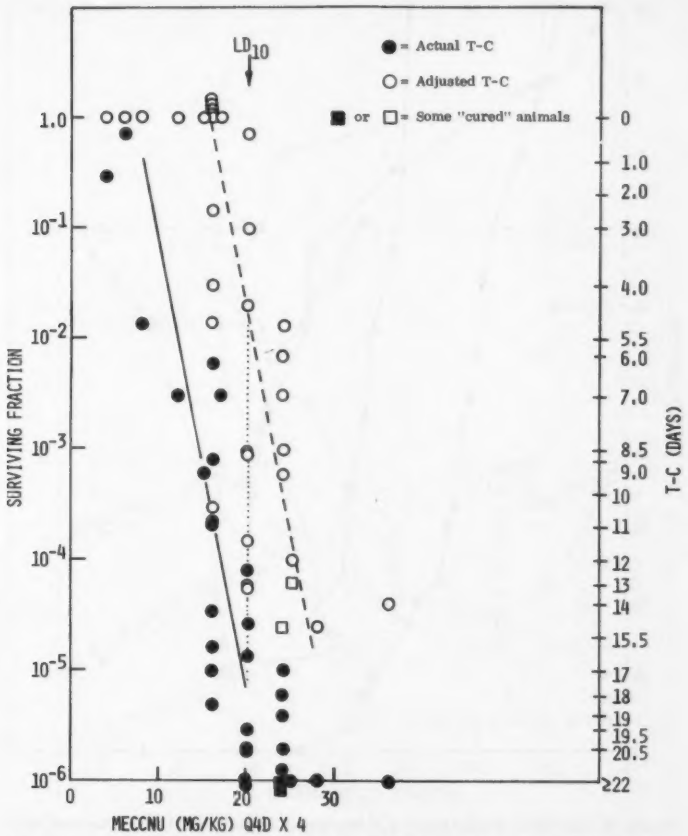

FIGURE 9.—Estimate of cell survival of sc implanted (trocar fragments) B16 melanoma after treatment with multiple doses of methyl-CCNU. Surviving fraction is based on observed T-C in relation to historical growth curve (*from* fig 1); 10 mice/data point. Adjusted T-C = actual T-C less the number of days between the first and last treatment during which time "some" tumor cell repopulation may have occurred. The dotted line indicates the surviving fraction range, depending on interpretation.

purposely pooled (unstaged—comparable to a clinical setting where the extent of tumor burden may not be precisely known) and treated with methyl-CCNU, 30% of the mice were "cured." But when that unstaged group was broken down into its component parts, it can be seen that the major effect of treatment, in terms of "cure rate" was in the 10^3-cell group (70% tumor-free survivors) and that no "cures" were obtained in the 10^7-cell group. When cyclophosphamide was added to the treatment regimen (fig 11), "cure" increased by about 10% in the unstaged group but to 90% in the 10^3-cell group, and from about 10% to 40% in the 10^5-cell group.

These results suggest that chemotherapy, as an adjuvant to surgical treatment, may be aimed at eradication of tumors in those groups where tumor-cell burdens are relatively small. The approach, from a regimen design standpoint, may be quite different from that used solely to provide increased survival time where low-dose maintenance therapy may suffice.

Table 7 shows the response of the sc B16 melanoma to a single LD10 dose of methyl-CCNU as a function of tumor size that ranged from 10^3 cells to 1.0 g (10^9 cells) or more. Using the criterion of percent of responders, methyl-CCNU was no more effective against this advanced mouse tumor than it is against melanoma in man (although, as shown earlier, that endpoint does not adequately reflect the percent cell kill obtained). Using "curability" as the criterion of activity, one can see a clear relationship between tumor burden and response to treatment, suggesting that agents that do not yield large percentages of responders among large, advanced tu-

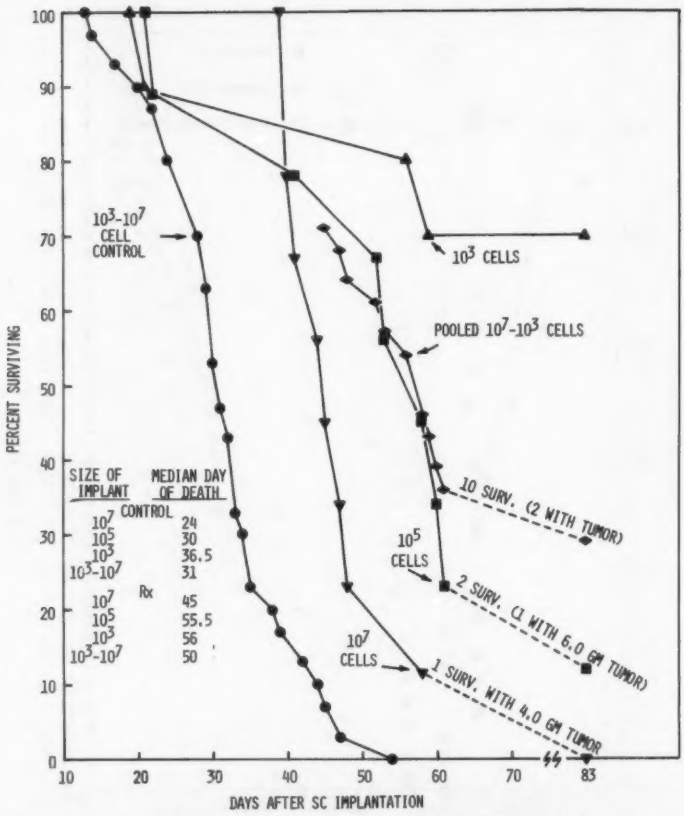

FIGURE 10.—Survival of mice bearing sc implanted 10^7, 10^5, or 10^3 B16 melanoma cells with lethally irradiated feeder cells (see legend, fig 1) after treatment with methyl-CCNU, 20 mg/kg/day on Days 1,5,9,13; 10 mice/group (30/pooled group).

TABLE 7.—Effect of tumor staging on curability: melanoma and methyl-CCNU

	Mouse* (%)	Man† (%)
Advanced tumor (≥ 10^9 cells)		
Percent responders	13	13
Curability	0	
Fragment or 10^7 cells		
Curability	4	
10^5 Cells		
Curability	18	
10^4 Cells		
Curability	33	
10^3 Cells		
Curability	70	

*Total experience: response of sc implanted B16 melanoma to methyl-CCNU, 40 mg/kg, single dose.

†From Program Logic and Scientific Reports. *In* Report of the Division of Cancer Treatment, NCI, 1974. Bethesda, Md, NCI, Sept 1974, vol 2.

mors should not necessarily be discarded as inactive. Such chemotherapeutic agents may be useful in surgical adjuvant settings where the tumor burden may, in some groups, be relatively low.

The effect of staging on surgical "cure" has been previously documented (14). The relationship for C3H mammary carcinoma is shown in figure 12. Where the results of both surgical treatment and chemotherapy are independently improved by proper staging, it stands to reason that the combination of the two modalities should offer even better "cure" yields in the properly staged disease.

It has been the intent of this paper to show that selections of particular therapeutic regimens and the prediction of their success or failure can be based on four, seemingly elementary, pieces of information. These are: (*a*) overall average tumor volume doubling time; (*b*) tumor cell kill per dose or course; (*c*) host toxicity (ie, normal tissue recovery time); and (*d*) tumor stage. While this approach may seem overly simplistic, or even naïve, the application of

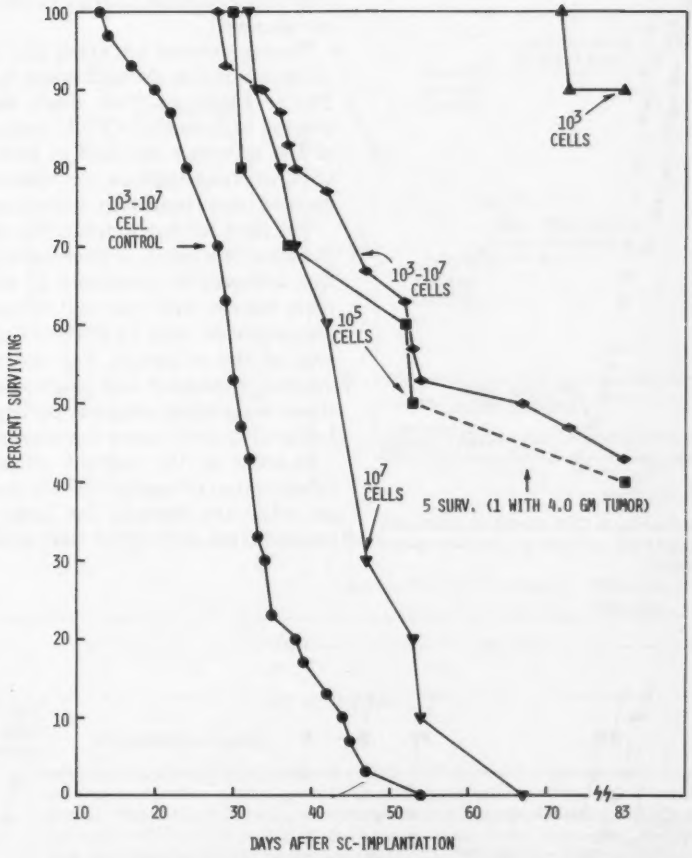

FIGURE 11.—Survival of mice bearing sc implanted 10^7, 10^5, or 10^3 B16 melanoma cells with lethally irradiated feeder cells (see legend, fig 1) after treatment with methyl-CCNU, 20 mg/kg/day on Days 1,5,9,13, plus cyclophosphamide, 7.0 mg/kg/day qd for 1-15 days; 10 mice/group (30/pooled group).

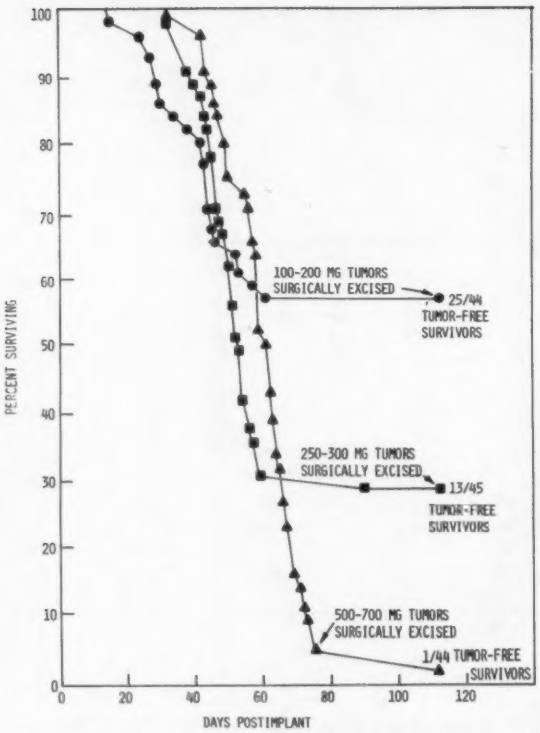

FIGURE 12.—Survival of mice bearing sc C3H mammary adenocarcinoma after surgical excision of the sc tumor at various stages of growth [data of T. H. Corbett].

this information in surgical/chemotherapeutic trials in mice bearing advanced, otherwise lethal tumors has resulted in "cure" of some percentage of those tumor hosts.

Previously it has been shown that improved "cure rates" could be obtained in animals bearing advanced tumors of the lung (10,14) and the breast (30). We now show that similar improved results may be obtained in animal tumors of the colon and a melanoma as well.

The response of advanced B16 melanoma to surgical excision plus chemotherapy is shown in figure 13. The sc tumor at that stage was incurable when treated with methyl-CCNU alone. Surgical excision of the sc tumor resulted in 20% tumor-free survivors, whereas combination treatment yielded 70%, a marked improvement in "cure rate."

The need for early, intensive therapy is indicated in figure 14 where it is shown that a 90% "cure rate" was achieved by treatment of advanced C3H mammary tumors with maximal tolerated doses of cyclophosphamide plus CCCNU-*cis* after surgical excision of the sc tumor. Yet under identical circumstances, treatment with approximately two thirds of those doses after surgical excision resulted in little better than 50% tumor-free survivors.

Because of the current clinical interest in the combination of methyl-CCNU plus 5-FU as a surgical adjuvant therapy for large bowel cancer, we examined the activity of that combination against a

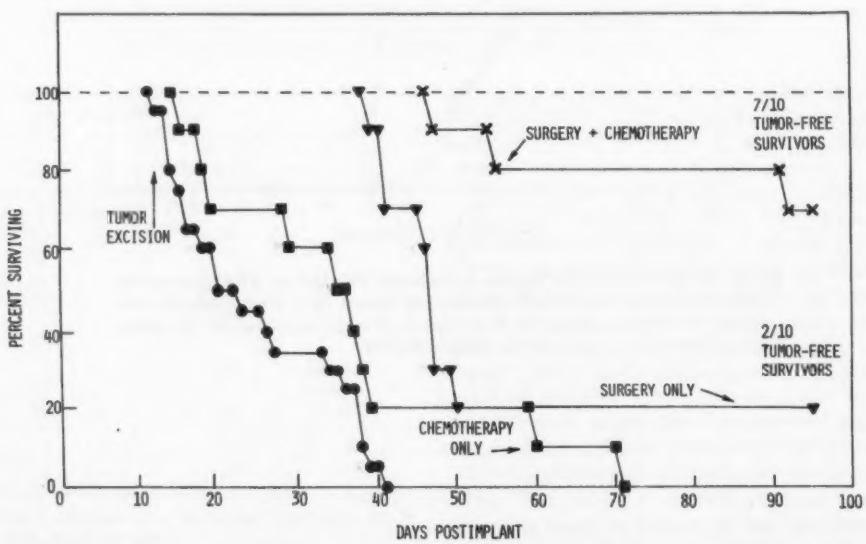

FIGURE 13.—Survival of mice implanted sc with B16 melanoma (trocar fragments) after surgical excision alone of the primary sc tumor (tumor size at time of surgery = 0.9-1.1 g), treatment with methyl-CCNU alone (36 mg/kg, single dose), or surgical excision with chemotherapy 1 day later [data of D. J. Dykes].

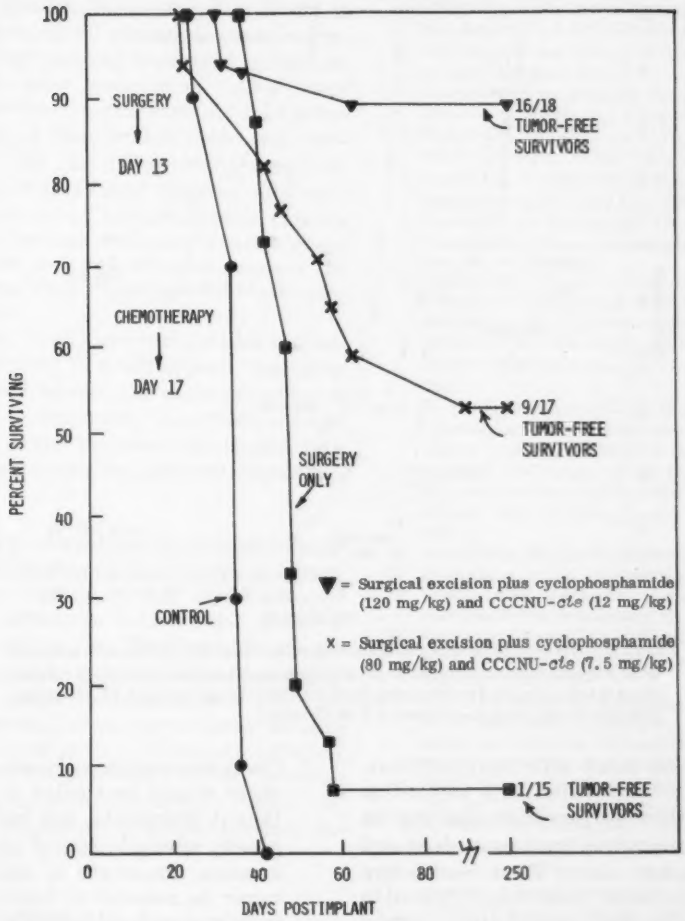

FIGURE 14.—Survival of mice implanted sc with C3H mammary adenocarcinoma (trocar fragments) after surgical excision alone of the primary sc tumor (tumor size at time of surgery about 750 mg), or surgical excision plus cyclophosphamide (120 mg/kg) and CCCNU-*cis* (12 mg/kg) in single simultaneous doses, or surgical excision plus cyclophosphamide (80 mg/kg) and CCCNU-*cis* (7.5 mg/kg) in single simultaneous doses [data of T. H. Corbett].

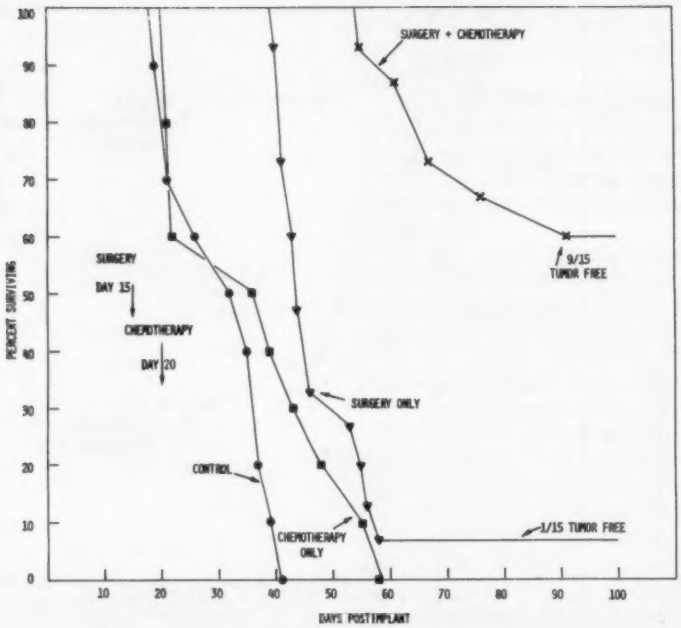

FIGURE 15.—Survival of mice implanted sc with colon tumor No. 26 (trocar fragments) after surgical excision alone of the primary sc tumor (tumor size at time of surgery about 0.8 g) or surgical excision plus methyl-CCNU (18 mg/kg) and 5-FU (36 mg/kg) in single simultaneous doses [data of T. H. Corbett].

metastatic mouse colon tumor after surgical treatment (fig 15). In this single trial, 60% tumor-free survivors resulted from surgery/chemotherapy as opposed to 7% from surgical treatment alone and zero from chemotherapy alone. These results are about twofold or more better than those achieved in other studies[8] using the single agents after surgical treatment. It was possible, therefore, to improve on initially obtained results. This and future improvement in therapy will depend not only on the proper application of chemotherapeutic principles but on correct selection of agents for combination.

CONCLUSIONS

In conclusion, I would suggest that:

1. In spite of the criticism that experimentalists, with their animal tumors, have *only* validated clinical successes, it would seem necessary to at least validate the animal tumor in terms of growth, metastatic, and chemotherapeutic response characteristics in order to authenticate it as a "model."
2. Chemotherapeutic response of the animal tumor model should be similar to that of the human type it represents, but because of the heterogeneity among tumors of both animals and man, it seems unrealistic to expect a single animal tumor to respond to treatment in exactly the same way as do all tumors of a particular type in man.
3. Endpoints commonly used as measures of chemotherapeutic success should be qualified and, when possible, converted to estimates of surviving fractions of the tumor population; where dose-fraction/cell-survival data may be useful in predicting optimal treatment regimens, intermediate endpoints, such as percent responders, may, in certain circumstances, be quite misleading.
4. Tumor volume doubling times, based on measurements made in the latter stages of growth, are not particularly meaningful, especially when used to estimate rates of recurrence. Overall volume doubling times seem to more closely parallel recurrence rates from cell populations that have been reduced by treatment.
5. The stage of tumor growth, both primary and metastatic, has a direct bearing on surgical and chemotherapeutic curability, at least among

[8]Corbett TH, Southern Research Institute. Unpublished data.

these animal tumors. Based on a few pieces of information considered essential, experimental trials employing surgical treatment and chemotherapy have been designed to "cure" large percentages of mice bearing several tumor types including those of lung, breast, colon, and a melanoma. While this may be an overly simplistic approach, the fact remains that animals have been cured of advanced, otherwise lethal cancers using agents that are clinically available and which alone are no more effective against the advanced animal tumor than against their counterparts in man.

6. If experimental results are translatable to man, even if only broadly, it would appear that using predictive tumor models, the experimentalist is in a position to determine quantitatively and with some reasonable assurance of success, optimal approaches to surgical adjuvant chemotherapy.

Surgical adjuvant chemotherapy is a promising tool for the clinical oncologist. But the promise rests on careful disease staging, proper application of chemotherapeutic principles to treatment, and precise quantitation of results. The "cured" animals presented in this paper were those whose tumor cell populations after surgical treatment were relatively small. It is likely that currently projected clinical "cures" fall in the same pattern. If experimentalists are to offer leads to the clinician that hold promise of improving surgical/chemotherapeutic results, we must find means of destroying larger residual tumor cell populations.

LIST OF COMPOUNDS

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8

CCCNU-*cis*: NSC-153174; CAS reg. No. 42558-93-0; cyclohexanecarboxylic acid, 4-[3-(2-chloroethyl)-3-nitrosoureido]-, *cis*-

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

5-Fluorouridine: NSC-27640; 5-fluoro-2'-deoxyuridine; 5-FUDR

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,-11,20-trione, 17,21-dihydroxy-

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Cell Survival Systems for the Evaluation of Irradiation and Drug Combinations[1]

Vincent H. Bono, Jr.,[2] John E. Byfield, [3] Ronald F. Hagemann,[4] Samuel H. Lesher,[4] Frederick A. Valeriote, [5] and Teresa Vietti[5,6]

Two kinds of chemical agents have been studied in combination with irradiation. One type of agent has little cytotoxic activity but increases the sensitivity of the target cell population to irradiation. These agents usually exert their effects by increasing the lifetime and effectiveness of the free radicals produced by irradiation. A second type of agent is the active cancer chemotherapeutic drug which may potentiate the action of local irradiation but also produces cytotoxic effects elsewhere. These latter drugs may act by interfering with X-ray-damage repair (eg, repair excision, repair replication, and repair ligase activity) or by synchronizing proliferating cells, or they may interact in ways yet unknown. Also, irradiation may increase cellular permeability and allow more drug to enter the cell. In addition, the evaluation of the combined action of irradiation and chemical agents should take into consideration selectivity of action with respect to host and tumor cells, although this is of greater importance when considering two-drug combinations that act systemically. Therefore, the change in sensitivity of the normal host cells needs to be evaluated in parallel with that of the tumor cells.

Cell Culture Systems

The simplest system for evaluation of drug-irradiation interaction is by cell culture. The environmental conditions can be closely controlled and reproduced: X-rays and drug-dose level, exposure time, dose interval, and sequence of exposure can be accurately measured. Cell survival is determined by an assay for the proliferative capacity of each cell—the most relevant parameter for both cancer-cell and normal-cell renewal systems. In addition, the growth kinetics of the exposed cells can be selected for examination, such as exponential (proliferating) or stationary phase (nonproliferating) cells.

During the past year, one of us (Dr. Byfield) has been using such a system to evaluate the actions of bleomycin, cyclophosphamide, and adriamycin alone and in combination with irradiation.

The effect of the simultaneous administration of bleomycin and irradiation on HeLa cells is shown in figure 1. The open circles represent the response to

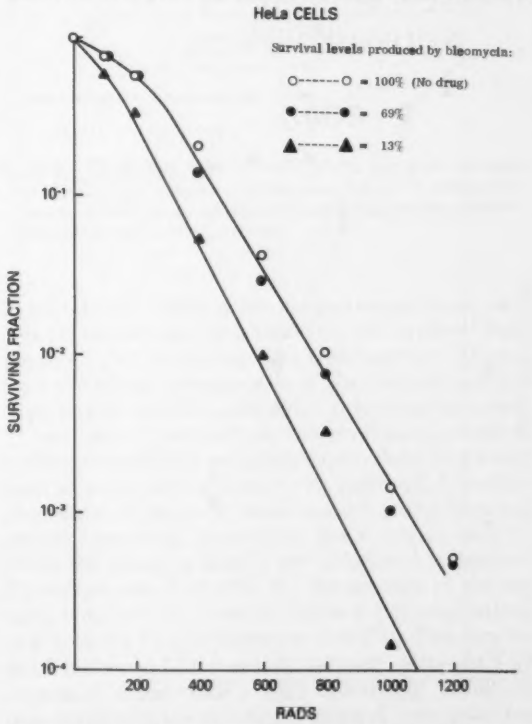

FIGURE 1.—HeLa cells were treated with the indicated radiation dose level with the simultaneous addition of bleomycin at concentrations which alone reduce survival to several levels. Nonirradiated controls and nonirradiated bleomycin-treated cells were both normalized to 100% survival.

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irradiation alone. The closed triangles represent the effect of irradiation on cells that have been exposed to a dose of bleomycin large enough to allow only 13% of the cells to survive. The results are normalized to the surviving fraction obtained with drug administration alone. The cells surviving after the administration of bleomycin are more sensitive to irradiation primarily because of a decrease in the extent of the shoulder region rather than because of a change in the slope of the dose-survival curve. A lower dose of bleomycin, which allows 69% survival (closed circles), did not alter the sensitivity of these cells to irradiation. The sequence of exposure to bleomycin and irradiation was not significant (fig 2) for a dose of bleomycin which allowed 70%-80% cell survival.

Some interesting effects are seen in studies on the CX-1B cell line, which is deficient in X-ray excision repair and is more sensitive to bleomycin than HeLa cells. At a dose level of bleomycin which allowed 70% cell survival, augmented sensitivity to irradiation was seen but only with prolonged exposure to the drug (fig 3). Exposure to bleomycin for 4 hours, beginning 1 hour before irradiation, produced the same

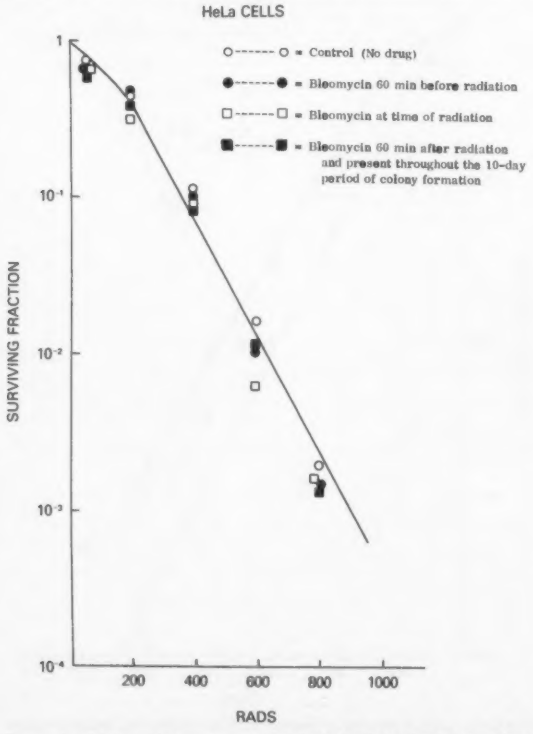

FIGURE 2.—HeLa cells were treated with the indicated radiation dose level and with bleomycin at a concentration which reduces survival to 70%-80%. Nonirradiated controls and nonirradiated bleomycin-treated cells were both normalized to 100% survival.

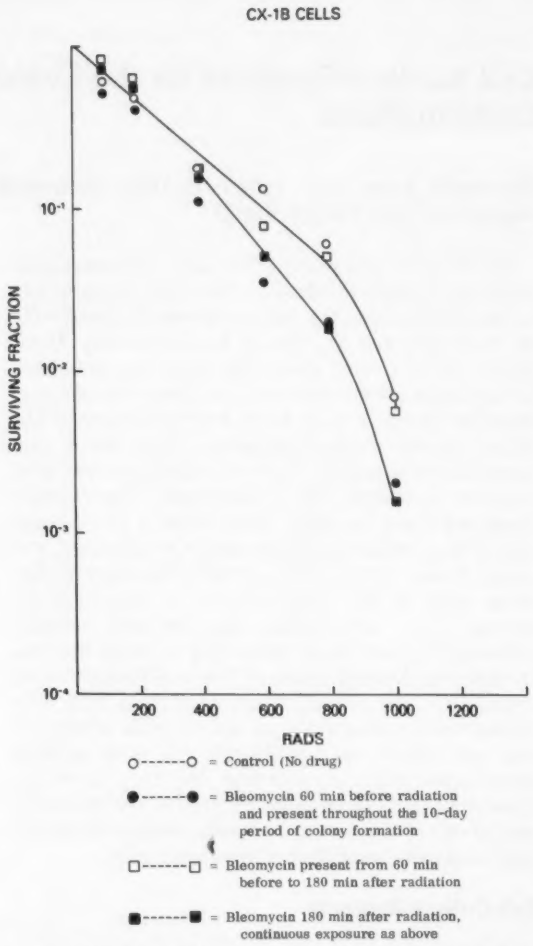

FIGURE 3.—CX-1B cells were treated with the indicated radiation dose level and with bleomycin at a concentration that reduces survival to 70%. Nonirradiated controls and nonirradiated bleomycin-treated cells were both normalized to 100% survival.

extent of cell kill but did not increase irradiation sensitivity. When exposed to the drug continuously, however, starting either before or after irradiation, the surviving cells were more sensitive. This suggests that accumulated net bleomycin damage may mediate the enhanced sensitivity to irradiation.

Similar studies have been done with peroxycyclophosphamide, an analog of cyclophosphamide which does not require microsomal activation. The survival of CX-1B cells at dose levels of peroxycyclophosphamide which alone reduce survival to either 75% or 9% is considerably reduced over that for irradiation alone. The maximum difference, however, is less than a factor of ten (fig 4). Adriamycin (fig 5) has a similar effect on irradiation sensitivity.

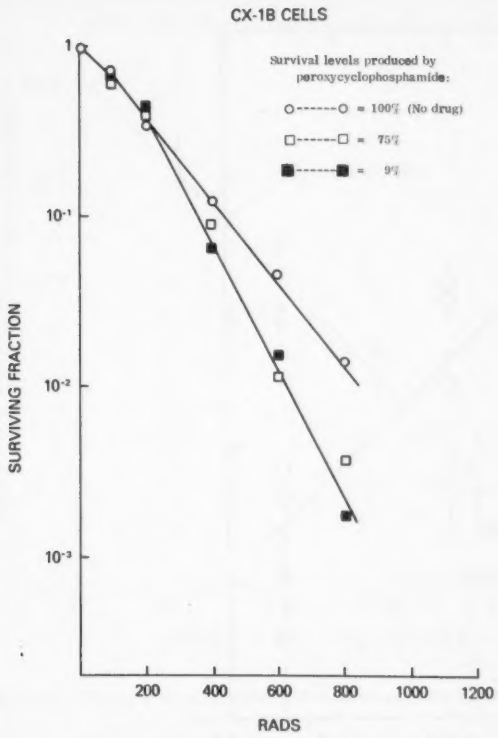

FIGURE 4.—CX-1B cells were treated with the indicated radiation dose level along with the simultaneous addition of peroxycyclophosphamide at concentrations which reduce survival to several levels. Nonirradiated controls and nonirradiated drug-treated cells were both normalized to 100% survival.

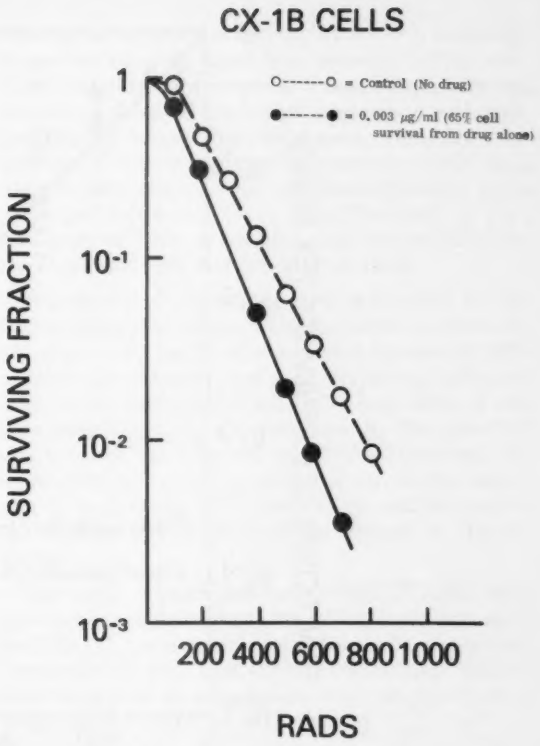

FIGURE 5.—CX-1B cells were treated with the indicated radiation dose level along with the simultaneous addition of adriamycin. Nonirradiated controls and nonirradiated drug-treated cells were both normalized to 100% survival.

For all three drugs, the augmented effect of irradiation was significant but probably represents independently exerted cytotoxic events in view of the significant differences in mode of action of the three agents. This does represent an advantage, but it cannot be taken as evidence which would predict significant therapeutic augmentation for these drugs since corresponding increased killing of normal cells might also occur. In addition, pharmacokinetic factors may influence the overall results.

In Vivo Systems

In vivo clonogenic assays analogous to that described above have been established for many tumor lines (1). Two of us (Drs. Valeriote and Vietti) have employed a spleen colony assay technique to examine the cytotoxic effect of drug combinations. $CD2F_1$ mice bearing an advanced L1210 leukemia or AKR mice bearing an AKR leukemia are treated with drugs singly at various dose levels and when either irradiation and drug or two drugs are examined, various sequences and time intervals are studied. Subsequently, at an appropriate time interval the animals are killed, a suspension of the femoral marrow is prepared, and this is injected into recipient mice. The number of macroscopic tumor colonies produced in the spleens of the recipient mice is determined and used as a measure of tumor-cell survival. A similar procedure is followed when assaying the hematopoietic stem cells except that the recipient host in which the assay is done is pre-irradiated to destroy its endogenous stem cells (2). An example of the results obtained is shown in figure 6 for irradiation, and in figure 7 for 5-fluorouracil (5-FU). The survival of leukemic L1210 colony-forming units (LCFU) decreases exponentially with increasing radiation dose levels and there is no evidence of a shoulder region. Similarly, the survival of AKR LCFU decreases exponentially with increasing dose levels of 5-FU (3). To evaluate the combined effect, a dose of radiation and 5-FU was used such that each reduced cell

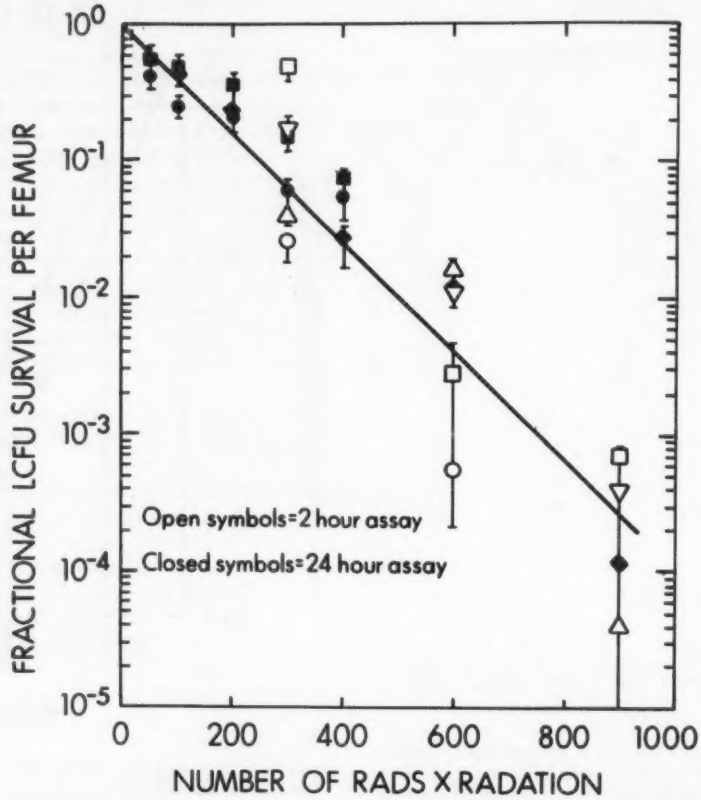

FIGURE 6.—Survival of LCFU after in vivo exposure to the indicated radiation dose level. Different symbols represent different experiments. Errors are ±SE.

survival to between 1% and 5%. The sequence of drugs and the time interval between them were varied, and the fractional survival of leukemic cells observed is shown in figure 8. We have defined the additive effect as the product of the surviving fractions of the single agents. It can be seen that the sequence and time intervals of administration made a significant difference in cell survival varying over 100-fold. When 5-FU was administered in the first few hours after irradiation, maximum cell kill was achieved. Similar irradiation combination studies are in progress with adriamycin, cytosine arabinoside, and other drugs.

Much of the work done using this system, however, has been with two-drug combinations and will be presented in detail elsewhere. Two examples will be given here since they show the kind of information that can be obtained. The interaction of 5-FU with melphalan is shown in figure 9. Maximum killing of L1210 LCFU with minimal killing of normal hematopoietic colony-forming units (NCFU) occurred when melphalan was administered 20 or more hours after 5-FU although LCFU destruction was essentially greater than additive throughout the interval range studied. This indicates the possibility of therapeutic advantage for this schedule, and studies testing this are being carried out. The combination of adriamycin and methotrexate shows no sequence or time interval dependence (fig 10) although LCFU killing is greater than additive throughout the interval studied. The LCFU were more sensitive to the combination than the NCFU.

Other combinations such as melphalan and adriamycin or cyclophosphamide and adriamycin show no sequence or time interval dependence for LCFU but yield additive cell killing. Some combinations are strongly interval-dependent such as cytosine arabinoside and daunorubicin (4) or 5-FU and adriamycin.

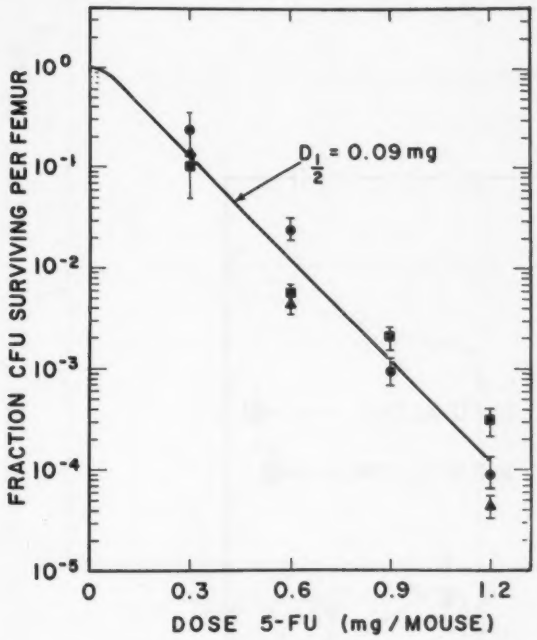

FIGURE 7.—Survival of LCFU after in vivo treatment with indicated dose levels of 5-FU.

Combined In Vivo and In Vitro Systems

A third system uses a combination of in vivo and in vitro methods. Tumor-bearing mice (P815X2 mastocytoma) are treated with drug and irradiation, and the survival of the tumor cells is determined in vitro by giving them an opportunity to form cell culture colonies under conditions where untreated tumor cells have a 90% efficiency for colony formation. In addition, the effects on the gastrointestinal tract are measured by determining the survival of jejunal crypts. Using methods developed by two of us (Drs. Hagemann and Lesher) (5) we have shown that gastrointestinal toxicity is primarily related to the extent of destruction of entire crypt units, rather than to the extent of crypt-cell death. This is due to the fact that the survival of one proliferating cell per crypt will result in the survival of the crypt.

The survival of jejunal crypts in mice treated with radiation is reduced to 1% at a dose of 1800 rads (fig 11). The simultaneous administration of either 5 or 10 mg/kg of adriamycin had no effect on the number of surviving crypts, although the extent of cell kill per crypt may be greater. The effects of adriamycin and irradiation on DNA synthesis in the jejunal mucosa are augmentative (fig 12). After a sublethal dose of irradiation, there is a decrease in DNA synthesis reaching a minimum at 1 day. This is followed by an overshoot as the crypts repopulate with cells and end up larger than they were originally. The addition of 5 or 10 mg/kg of adriamycin results in a prompt decrease in DNA synthesis followed by a prolonged depression for 3 days. The return to normal rates of DNA synthesis is not accompanied by the overshoot seen with irradiation alone.

The effects of adriamycin and irradiation on the tumor suggest an independent and additive effect (fig 13). A dose of 2 mg/kg of adriamycin kills about 50% of the 7-day-old tumor cells. At increasing radiation dose levels, adriamycin still kills only 50% of the surviving fraction, as evidenced by the parallel character of the survival lines. The 21-day-old tumor, which has a low growth fraction, is less sensitive to both adriamycin and X-rays, and the combination does not produce an advantage in the response.

The same results are seen when P815X2 cells growing in cell culture are studied in an in vitro system (fig 14). Once again adriamycin kills a constant percent of the cells that survive irradiation, and the surviving cells at the plateau level are much more resistant to both forms of therapy.

DISCUSSION

In summary, the systems described allow for an exact appraisal of the cell-killing effects of irradiation and drug under conditions of varying dose levels, sequences, and time intervals. Advantageous interactions can be clearly observed when present. While the in vitro systems have the advantage of controlability and are a homogeneous population, making biochemical and cellular studies relatively easy, in vivo systems can be employed to assess cytotoxic effects not only on tumor cells, but also on sensitive host tissues such as bone marrow and the gastrointestinal tract.

The results of these kinds of studies can be further pursued in two directions: first, toward more detailed mechanistic studies on how the molecular and cellular activities of the two agents interact with each other; and second, toward suggesting limits to the large range of the many variables present in a full in vivo therapeutic trial of two-agent combinations. To this latter objective an effective and open line of communication between the experimental cell biologist and the experimental tumor therapist must exist.

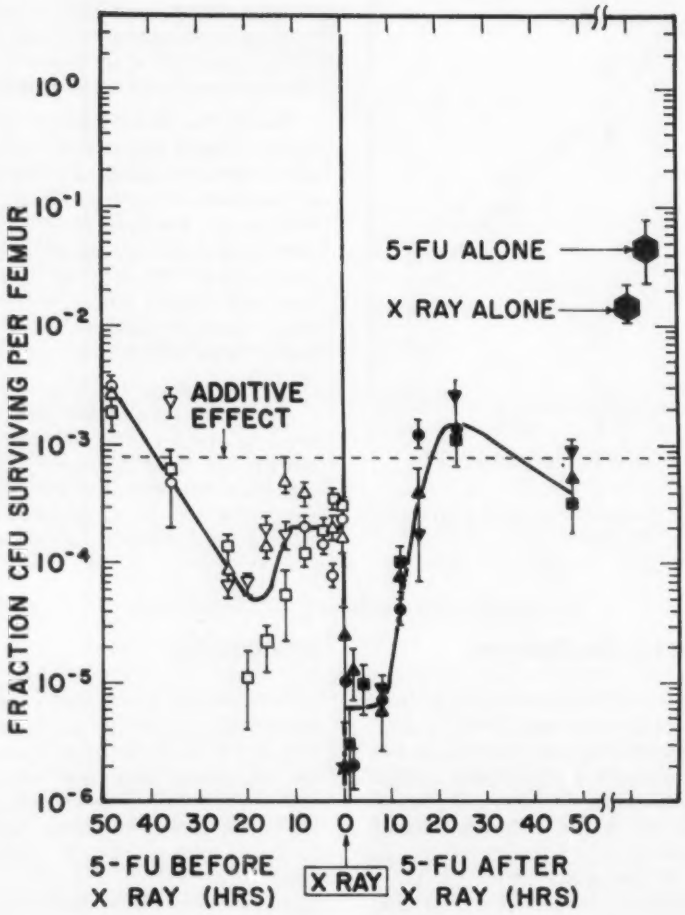

FIGURE 8.—Survival of LCFU in vivo following combination therapy with radiation and 5-FU administered in different sequences and time intervals.

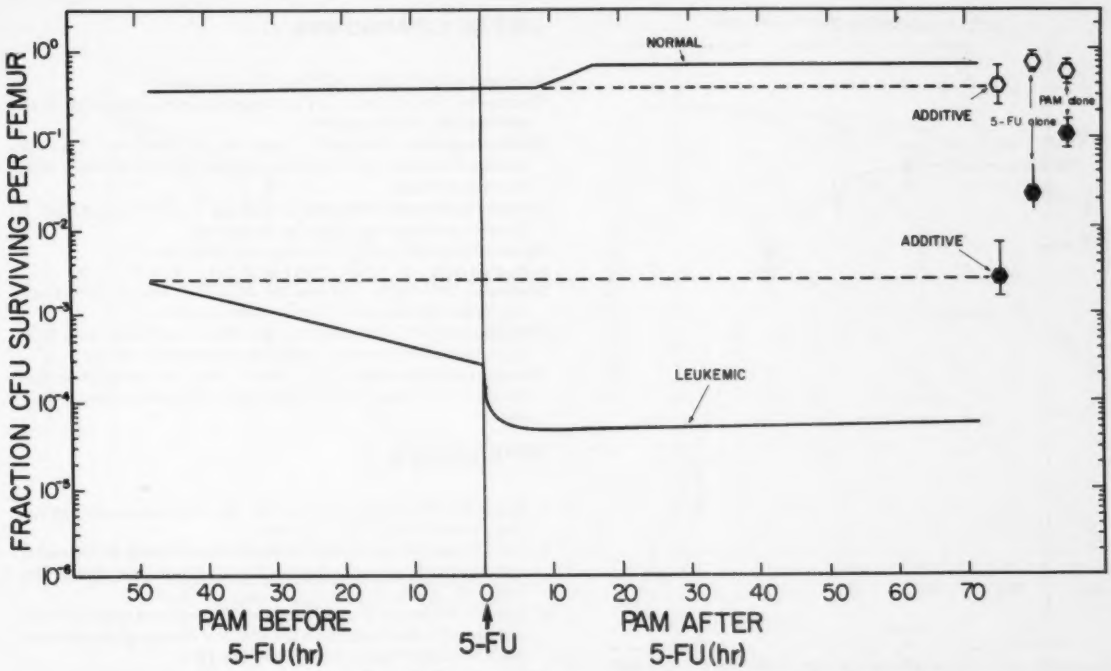

FIGURE 9.—Survival of NCFU and LCFU after in vivo treatment with 5-FU and melphalan (PAM).

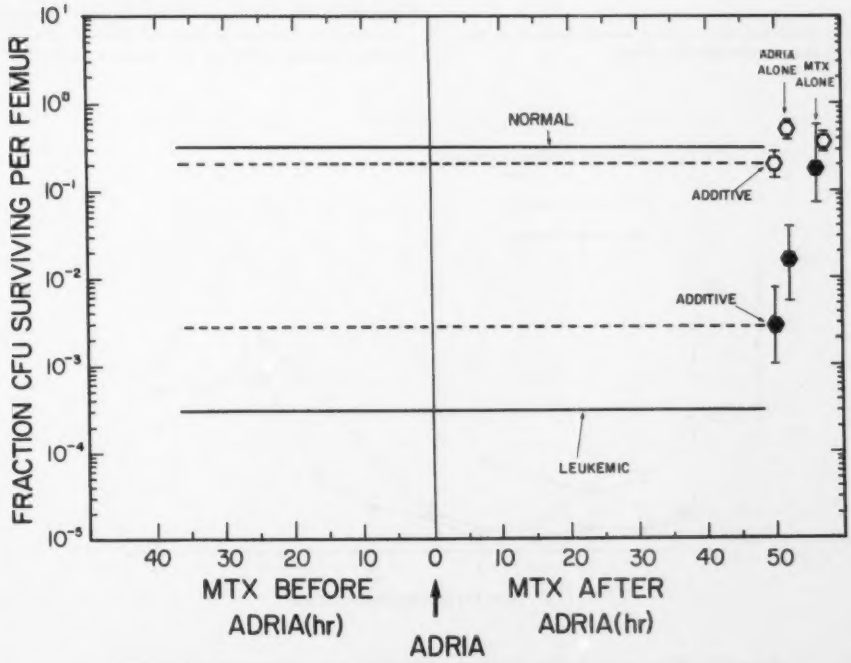

FIGURE 10.—Survival of normal hematopoietic stem cells (open symbols) and L1210 leukemic cells (closed symbols) after in vivo treatment with methotrexate (MTX) and adriamycin.

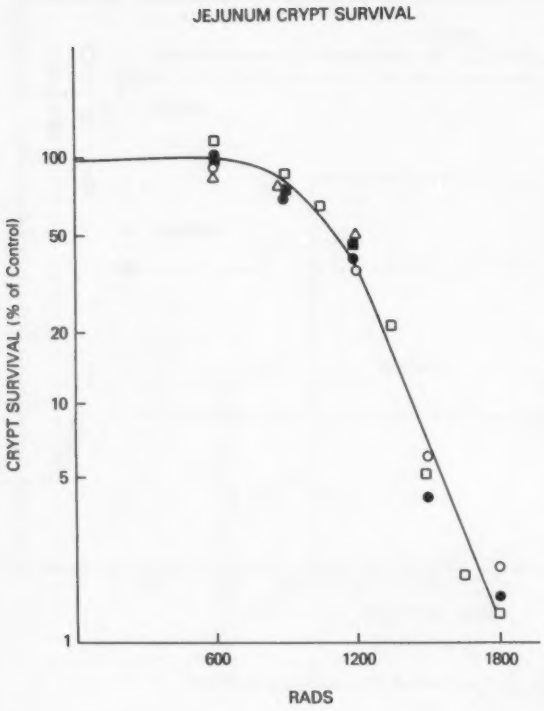

FIGURE 11.—Survival of jejunal crypts after administration of radiation at the indicated dose levels.

LIST OF COMPOUNDS

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8
Bleomycin: NSC-125066; [2,4′-bithiazole]-4-carboxylic acid, 2′-(2-aminoethyl)-, monohydrate
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate
Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride
Daunorubicin: NSC-82151; CAS reg. No. 23541-50-6
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU
Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-
Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-
Peroxycyclophosphamide: NSC-176986; 2*H*-1,3,2-oxazaphosphorin-2-amine, 4,4′-dioxybis[*N*,*N*-bis(2-chloroethyl)tetrahydro-, 2,2′-dioxide

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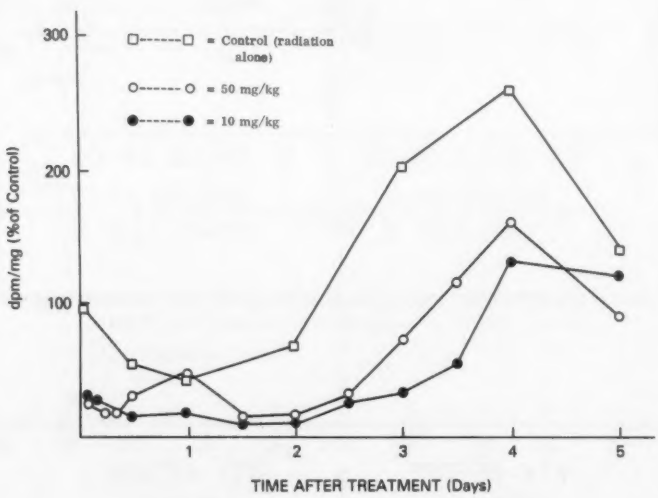

FIGURE 12.—Time course of DNA synthesis after treatment with a combination of radiation and adriamycin at varying dose levels. DNA synthesis was measured as the incorporation of ^{3}H-TdR/mg of wet wt.

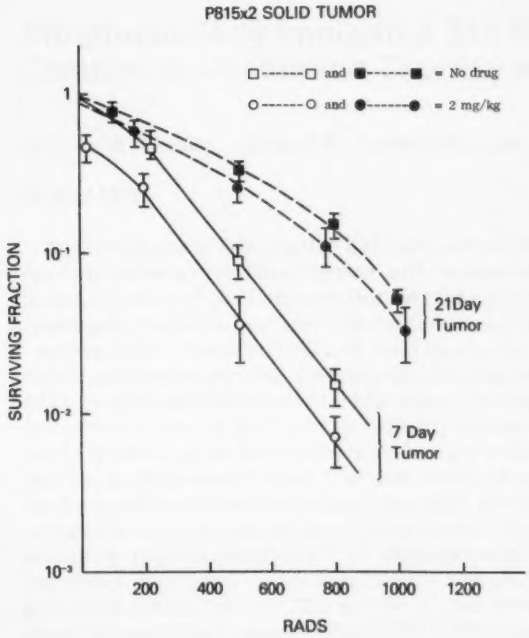

FIGURE 13.—Survival of P815X2 cells after in vivo treatment with indicated dose levels of radiation, with and without concurrent adriamycin. The surviving fraction is related to untreated tumor cell population for all samples.

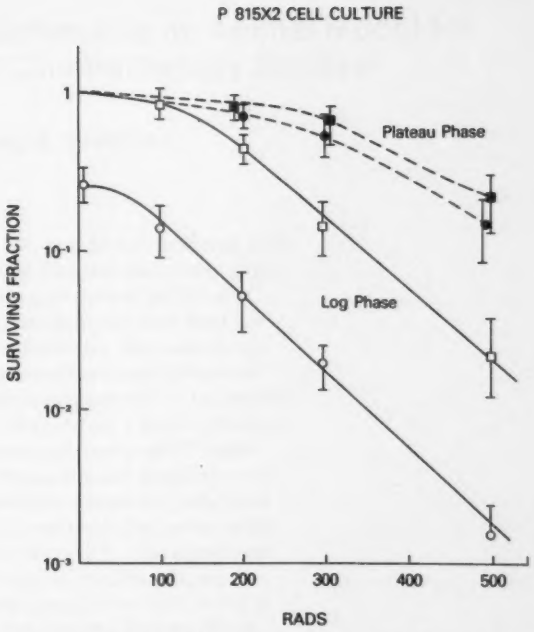

FIGURE 14.—Survival of P815X2 cells in culture after treatment as described in fig 13.

Intramuscularly Implanted B16 Melanoma as an Animal Model for Combined γ-Radiation Therapy and Chemotherapy Studies[1]

Isidore Wodinsky,[2] Joseph K. Swiniarski,[2] and John M. Venditti[3,4]

SUMMARY

An intramuscularly implanted B16 melanoma in the BDF_1 mouse was explored as a possible model for combined therapy with γ-radiation/drug. The B16 melanoma grew in accordance with the Gompertz function for solid tumors and showed an inverse relationship between host survival and tumor-cell inoculum, implying that host survival was a function of cell kill and could be used as a parameter for therapeutic activity. γ-Radiation given to the tumor-bearing leg in single or fractionated doses at 104 roentgens/minute showed that the tumor was sensitive as measured by tumor size and survival time. In the combined modality treatment, 5-fluorouracil and γ-radiation were administered on three different schedules and cyclophosphamide and γ-radiation on six different schedules. The therapeutic effect of the combined modality treatment was difficult to assess because the tumor growth could be completely inhibited by γ-radiation alone applied early in the disease (Days 1-5) and the local tumor could be made to regress completely if γ-radiation was given on Days 8-12. The combined modality treatments were not significantly superior to γ-radiation alone. Tumor growth was inhibited 55%-72% and the lifespans were increased from 65% to 102% above the untreated group and from 10% to 28% above γ-radiation alone by the administration of cyclophosphamide on Days 8-12 followed by γ-radiation on Days 15-19 at which time the local tumor weighed 3-4 g suggesting that the delayed drug/γ-radiation treatment schedule may be a useful model. Other quantitative assays are suggested and include assays to measure the time for the tumor to regrow to a standard size, assays to count the number of lung colonies, and assays to measure the size of the lung colonies at a specific time.

[Cancer Chemother Rep Part 2, vol 5:215-224, 1975]

A large number of both in vivo and in vitro experimental models have been developed to investigate the effect of combined γ-radiation and chemotherapy on neoplastic cells and to determine if the models could have predictive clinical value. The utilization of γ-radiation of solid tumors is well established. It acts on tumor cells that are located within the irradiated area and is dose limited by the toxicity to normal tissues in the applied area. Chemotherapy is effective systemically, killing tumor cells anywhere in the body, and is dose limited by bone-marrow aplasia, and intestine, liver, and kidney damage. Thus, the combined modality approach where γ-radiation would provide good local control and chemotherapy would permit the eradication of metastatic disease either undetected or unresectable seemed logical provided that the toxic effects were not additive and the combined treatment was tolerated by the host.

Despite the large number of models, there is still a compelling need for evaluating experimental models which would be useful prospectively rather than retrospectively in the design of combination regimens for the clinic.

In this study, we evaluate the use of γ-radiation therapy and chemotherapy on a localized intramuscularly (im) implanted B16 melanoma in the mouse. This model allows for the assessment in a quantitative manner by the methods of tumor size or animal survival.

MATERIALS AND METHODS

Neoplasm.—The B16 mouse melanoma was obtained initially from the Roscoe B. Jackson Memori-

[1]Supported by contracts N01-CM-33727 and N01-CM-53770 from the Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

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[4]*Reprint requests to:* Isidore Wodinsky, Arthur D. Little, Inc., Acorn Park, Cambridge, Mass 02140.

al Laboratory at Bar Harbor, Maine, in 1967. The tumors were subsequently carried in C57BL/6 mice of both sexes as subcutaneous transplants and were harvested for use 10-12 days after inoculation. For these studies, the tumor was homogenized in a Potter-Elvehjem tissue grinder and 0.25 ml of a 1:10 (wt/vol) tumor brei was inoculated into the gastrocnemius muscle of the right hind leg of both male and female BDF_1 mice (male C57BL/6 × female DBA/2). The tumors became palpable after 6 days and increased in weight exponentially until Day 13 at which time the growth rate decreased in accordance with the Gompertz function for solid tumors (fig 1). The B16 melanoma is lethal for the host and the range of days to death is narrow when the tumor is inoculated im. There was no difference in the median time of death of im inoculated B16 tumor-bearing mice for both sexes and there was an inverse relationship between median survival time and tumor cell inoculum (r = 0.998). The doubling time in the inbred host was calculated to be 43.1 hours (fig 2). Although the tumor grows well after an im inoculation, the metastatic rate to the lung, liver, and spleen, as bioassayed by the intracerebral inoculation of homogenates of these tissues into recipient mice or by direct examination of these organs, is

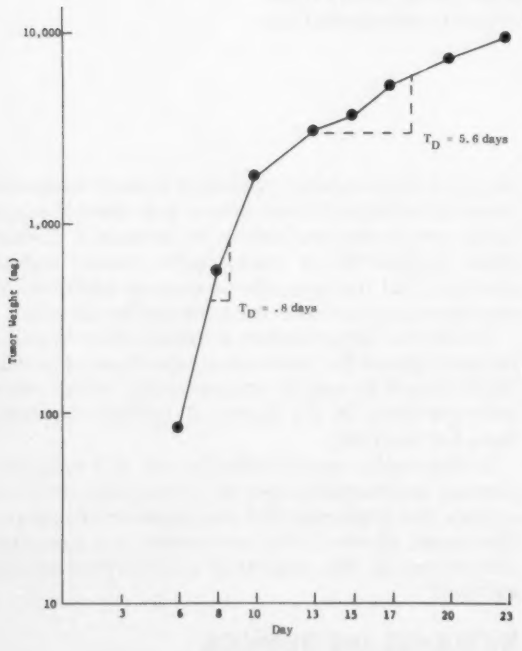

FIGURE 1.—In vivo growth of im implanted B16 melanoma in female BDF_1 mice.

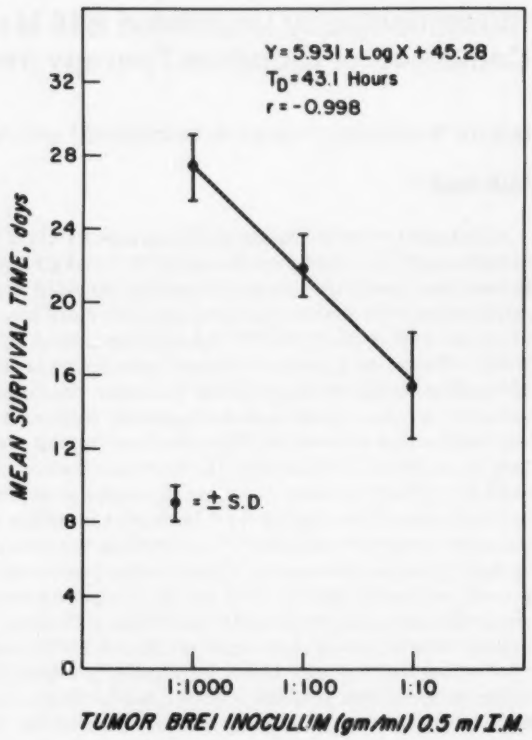

FIGURE 2.—Survival times of C57BL/6 mice inoculated im with B16 melanoma.

unpredictable However, all mice bearing im tumors eventually die from metastasis.

γ-Radiation.— γ-Radiation was delivered from a Tech-Ops ^{60}cobalt source with physical factors enabling a dose of 104 roentgens (R)/minute to be delivered to the tumor-bearing leg only at selected intervals following tumor inoculation. Dosimetry was checked with a Bendix dosimeter Model 686.

Treatment schedules.—The treatment schedules used in this study are presented in table 1. The actual doses and treatment schedules of γ-radiation and drug administered are shown in the appropriate tables and figures.

Tumor inhibition.—Caliper measurements of tumor length and width were made at weekly intervals starting on Days 15 and 22 in both the treated and control groups. Individual length × width measurements were obtained in square millimeters and a mean for each group was subsequently calculated.

Survival studies.—In addition to the above measurements, the mice were checked daily and deaths recorded. Increase in survival times of mice treated with the single or combined modality over untreated

TABLE 1.—Treatment schedules

Treatment schedule No.	Schedule of—		
	γ-Radiation	5-FU	Cyclophosphamide
1	Single dose, Day 7 only	—	—
2	Single dose, Day 13 only	—	—
3	Fractionated dose, Days 1-5	—	—
4	Fractionated dose, Days 1-10	—	—
5	Fractionated dose, Days 1-5	Days 8-12	—
6	Fractionated dose, Days 8-12	Days 1-5	—
7	Fractionated dose, Days 1-10	Days 1-10	—
8	Fractionated dose, Days 1-5	—	Days 1-5
9	Fractionated dose, Days 8-12	—	Days 1-5
10	Fractionated dose, Days 1-5	—	Days 8-12
11	Fractionated dose, Days 8-12	—	Days 8-12
12	Fractionated dose, Days 8-12	—	Days 15-19
13	Fractionated dose, Days 15-19	—	Days 8-12

control groups provided the basis for measuring the effectiveness of the treatment regimen.

RESULTS

Single-Dose γ-Radiation Studies (Day 7 Only)

On Day 0 female BDF_1 mice were implanted im with 0.25 ml of a 1:10 (wt/vol) homogenate of B16 melanoma cells in the gastrocnemius muscle of the right hind leg. The mice were randomized into groups of ten mice each. On Day 7 after inoculation, the mice were lightly anesthetized with Nembutal and arranged on lead shielding so that the tumor-bearing legs received a dose of 6075,4050,2700,1800,1200, 800, and 530 R at a rate of 104 R/minute while the dose rate to the body was 7.5 R/minute. On Day 10, half the mice in each group were killed. The rear legs were excised at the coxofemoral joint, and the tumor weights were calculated from the difference between the tumor-bearing right leg and the nontumor-bearing left leg of each mouse. The remaining mice were held for survival. At a dose of 6075 R of γ-radiation, the total whole-body dose received by the mice was 430 R.

The summary results of single-dose γ-radiation administered to the tumor-bearing leg only on Day 7 showed that the B16 melanoma was sensitive at doses of 1200-6075 R (table 2). With this treatment schedule, there was a 70%-81% inhibition of tumor growth on Day 10. There were also increases in survival times from 45% to >140% respectively with

TABLE 2.—Effect of single-dose γ-radiation on tumor inhibition and host survival of mice bearing B16 melanoma

Roentgen dose (Day 7)	Mean tumor weight* (g)	Tumor inhibition* (%)	Median survival time (days)	T/C† (%)	Mortality (%)
0	1.56	0	20.0‡	100	100
530	0.58	63	23.0§	115	100
800	0.89	43	29.0	145	100
1200	0.47	70	33.0	165	100
1800	0.41	74	43.0	215	100
2700	0.37	76	45.0	225	60
4050	0.30	81	45.0	225	60
6075	0.32	79	>48.0	>240	20

*Average of 5 mice on Day 10.
†T/C = treated animal tumor weight/control animal tumor weight.
‡Median of 25 mice for control group.
§Median of 5 mice for treated groups.

80% of the mice surviving at the highest dose. At the two highest doses (4050 and 6075 R) there was extreme local tissue damage with necrosis.

A second experiment was completed in which γ-radiation was applied to the tumor-bearing leg on Day 13 under conditions similar to the Day 7 experiment except that only doses of 800 and 1200 R were used. One half of the mice were killed on Day 16, their tumors were weighed, and the remainder were held for survival. The results showed that single doses of γ-radiation administered 13 days after tumor implantation at these levels did not inhibit tumor growth or increase median survival times significantly above the control group.

Fractionated Dose Studies of γ-Radiation

Groups of mice received five daily fractionated doses of γ-radiation at a level of 1200-200 R/day or at a level of 600-100 R/day × 10 starting on Day 1 after tumor inoculation.

The results showed that fractionation of the γ-radiation administered in five or ten equal daily doses produced a dose-dependent increase in median lifespan and in tumor inhibition. The 50% survival rate and the increase in lifespan (ILS) for both treatment schedules were similar and are shown in figures 3 and 4. This indicates that the increase was essentially due to the total dose of γ-radiation administered and that there were no significant differences between the two schedules. Tumor size was measured on Day 15 after treatment with 1000 R, and animals receiving treatment on Days 1-5 showed a 25% tumor inhibition while those receiving treatment on Days 1-10 showed a 43% tumor inhibition. At higher total doses (2000-6000 R) there were no differences between the two treatment schedules. By Day 22, the tumor inhibition produced on the treatment schedule of Days 1-10 was significantly lower

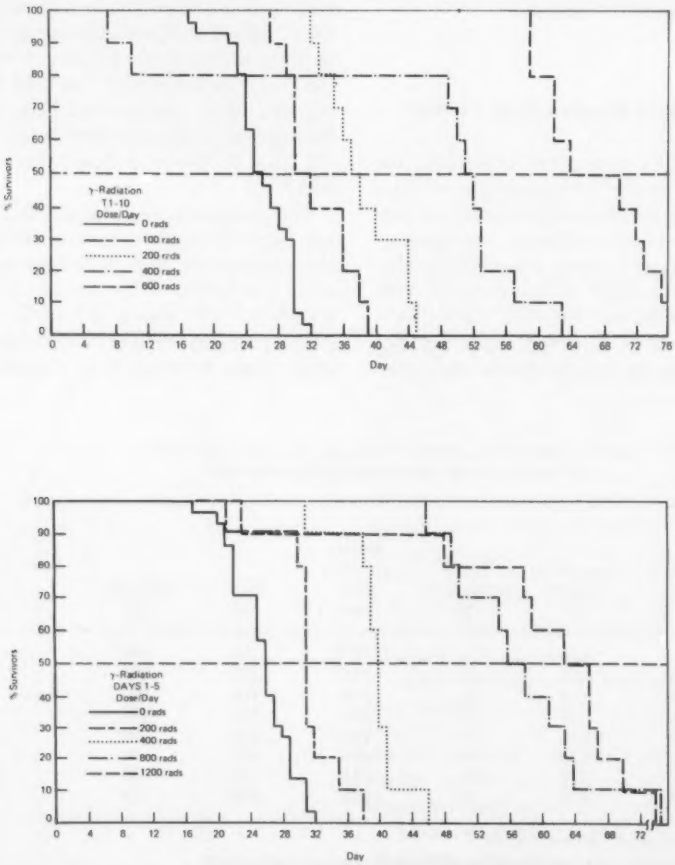

FIGURE 3.—Effect of fractional doses of γ-radiation on the survival of BDF_1 mice bearing im implanted B16 melanoma.

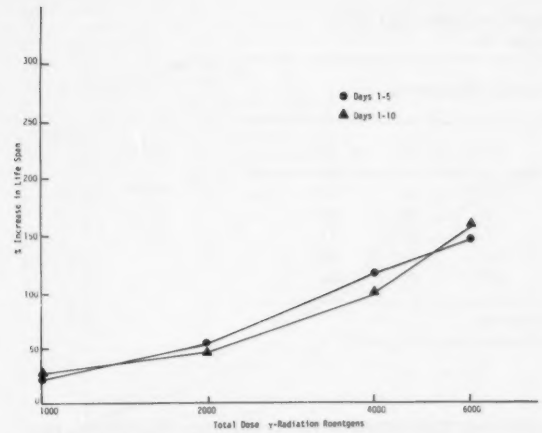

FIGURE 4.—Effect of fractional doses of γ-radiation on the lifespan of BDF_1 mice bearing im implanted B16 melanoma.

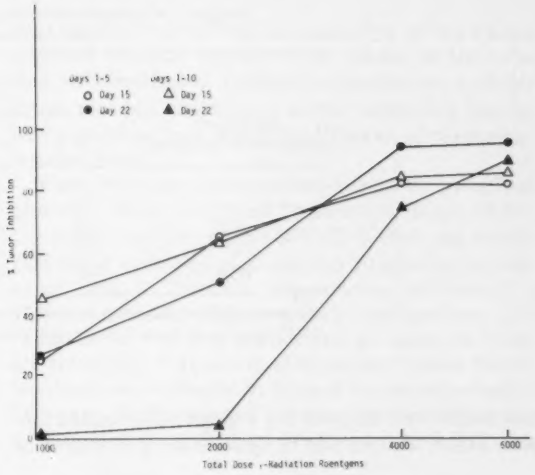

FIGURE 5.—Effect of fractional doses of γ-radiation on the tumor growth of im implanted B16 melanoma.

than on the treatment schedule of Days 1-5 at doses of 1000 and 2000 R. The inhibition was probably significant at the 4000 R dose, and at 6000 R no differences were noted (fig 5).

COMBINED MODALITY STUDIES

γ-Radiation and 5-Fluorouracil[5]

In the combined modality treatment, 5-fluorouracil (5-FU) was administered intraperitoneally (ip) on three schedules: (*a*) 5-FU was given daily on Days 1-5 followed on Days 8-12 by γ-radiation; (*b*) γ-radiation was administered on Days 1-5 × day followed on Days 8-12 by 5-FU; or (*c*) 5-FU and γ-radiation were given concomitantly on Days 1-10 with 5-FU given 1 hour after γ-radiation.

γ-Radiation, Days 1-5; 5-FU, Days 8-12.—5-FU given at doses of 7.5-60 mg/kg/day to tumor-bearing mice as a single agent was inactive and produced a tumor inhibition of 21% at the optimal dose of 30 mg/kg/injection by Day 22. When combined with γ-radiation, no meaningful differences in tumor inhibition were observed with doses of (*a*) 15 and 30 mg/kg of 5-FU and 1000-6000 R of γ-radiation, or (*b*) 7.5 mg/kg of 5-FU and 4000-6000 R of γ-radiation. Similarly, 5-FU alone did not increase the lifespans of the tumor-bearing mice significantly above the control group. At the optimal dose (30 mg/kg), there was a 17% ILS whereas γ-radiation alone produced substantial increases in lifespan at total administered doses of 2000 R and higher. At doses of 6000 R and 30 mg/kg of 5-FU, some toxic effects were noted by the slight decrease in lifespan (fig 6).

γ-Radiation, Days 8-12; 5-FU, Days 1-5.—This was the obverse experiment of the previous study. However, because the higher doses of γ-radiation had masked the effect of the combined treatment, lower doses were used in this experiment. The results shown in table 3 demonstrate that 5-FU alone was not effective against im implanted B16 melanoma on this schedule and γ-radiation was effective only at the highest dose administered. None of the combinations showed therapeutic potentiation. Measurement of the tumor growth by Day 22 showed no differences over that obtained with γ-radiation alone (fig 7).

γ-Radiation, Days 1-10; 5-FU, Days 1-10.—The results shown in figure 8 are similar to the previous experiments and again show that this schedule was not significantly different. Tumor inhibition and survival time were not superior to treatment with γ-radiation alone.

γ-Radiation and Cyclophosphamide[6]

Six therapy schedules have been completed where γ-radiation was combined with cyclophosphamide. Five of the schedules (figs 9-13) showed that almost total tumor inhibition could be obtained even if

[5]5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU. Supplied by DR&DP, DCT, NCI.

[6]Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,-3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan. Supplied by DR&DP, DCT, NCI.

TABLE 3.—Combined modality studies of γ-radiation and 5-FU: effect on lifespan (Day 43)

Radiation* (total dose in roentgens)	%ILS after 5-FU dose† of—				
	0	3.8	7.5	15	30
0	0	-4	4	4	7
250	11	6	7	15	20
500	22	24	13	13	11
1000	15	15	20	19	20
2000	41	31	48	37	39

*Treatment given Days 8-12 to tumor-bearing leg only.
†5-FU dose given in mg/kg/injection. Treatment given daily Days 1-5 ip.

treatment was delayed for 8 days after tumor implantation. The results in figure 9 showed that concomitant treatment with γ-radiation and cyclophosphamide was toxic for the mice at doses of 1200 R/day and 25 mg/kg/day × 5 respectively. However, lower doses of the combined treatment showed no differences in ILS from that obtained with γ-radiation alone, and the optimal ILS was 286% with three of ten long-term survivors.

The results in figure 10 show a slight decrease in ILS from the previous therapy schedule. The maximal ILS was 221% obtained with 1200 R of radiation and 3.1 and 12.5 mg/kg of cyclophosphamide.

On the next treatment schedule (γ-radiation, Days 1-5, and cyclophosphamide, Days 8-12) it was apparent that optimal ILS was obtained with γ-radiation alone at 1200 R (fig 11). This produced a 200% ILS above the control group. Combined drug/radiation treatment with 1200 R of γ-radiation produced a 117%-171% ILS.

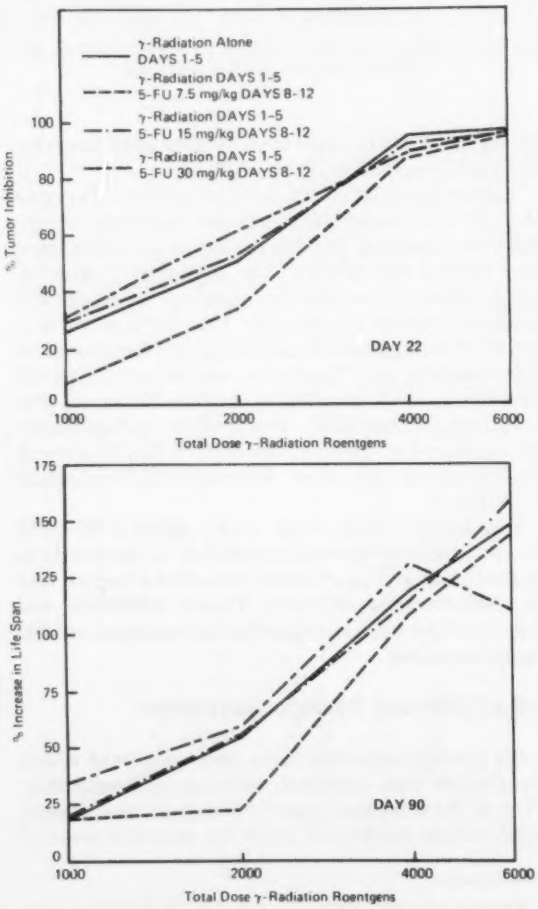
γ-Radiation Alone DAYS 1-5
γ-Radiation DAYS 1-5 5-FU 7.5 mg/kg DAYS 8-12
γ-Radiation DAYS 1-5 5-FU 15 mg/kg DAYS 8-12
γ-Radiation DAYS 1-5 5-FU 30 mg/kg DAYS 8-12
% Tumor Inhibition
DAY 22
Total Dose γ-Radiation Roentgens
% Increase in Life Span
DAY 90
Total Dose γ-Radiation Roentgens

FIGURE 6.—Combined modality studies of γ-radiation and 5-FU: effect on tumor growth and lifespan.

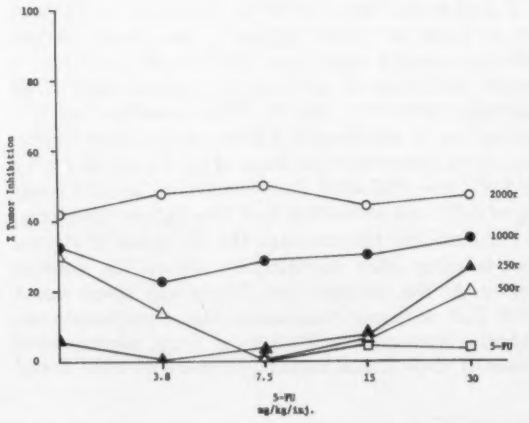

FIGURE 7.—Combined modality studies of γ-radiation (Days 8-12) and 5-FU (Days 1-5): effect on tumor growth (Day 22).

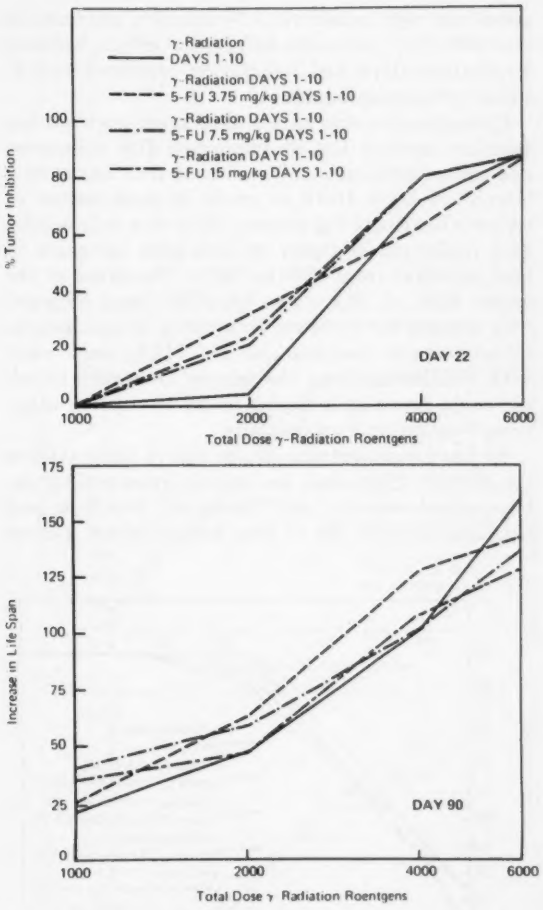

FIGURE 8.—Combined modality studies of γ-radiation and 5-FU: effect on tumor growth and lifespan.

One additional interesting observation on the treatment schedule was that the host was apparently able to tolerate a daily dose of 1600 R/day.

Figure 12 illustrates the results when cyclophosphamide and γ-radiation are given concomitantly on Days 8-12. The percent tumor inhibition was dose related and similar to that obtained by γ-radiation alone. The ILSs were also dose related and probably not significantly different from those obtained with γ-radiation alone, although the %ILSs were slightly higher for the combined modality at the 200-800 R γ-radiation dose levels.

The effect of γ-radiation given on Days 8-12 alone or combined with cyclophosphamide injected on Days 15-19 is shown in figure 13. The results were similar to the previous therapy regimens showing that the local tumor was still sensitive to γ-radiation and that the ILS was probably related to the total dose of γ-radiation. Cyclophosphamide as a single agent was not effective in either inhibiting the tumor growth or increasing the lifespan of the tumor-bearing mice.

When the obverse experiment was done (cyclophosphamide, Days 8-12, and γ-radiation, Days 15-19), the tumor inhibition was 55%-72% with the combinations of cyclophosphamide and γ-radiation at doses of 1200 R. Previous experiments had shown a 97%-100% tumor inhibition with combinations of γ-radiation at 800 R or higher and all doses of cyclophosphamide. This decrease in percent tumor inhibition was also reflected in a lower increase in median lifespans of the treated groups. Cyclophosphamide at a dose of 25 mg/kg/day produced a 50% ILS as a

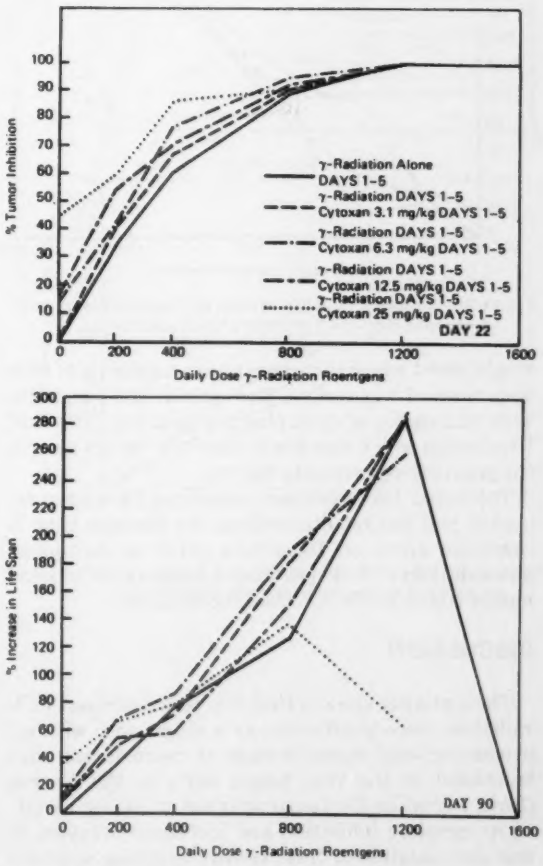

FIGURE 9.—Combined modality studies of γ-radiation and cyclophosphamide: effect on tumor growth and lifespan.

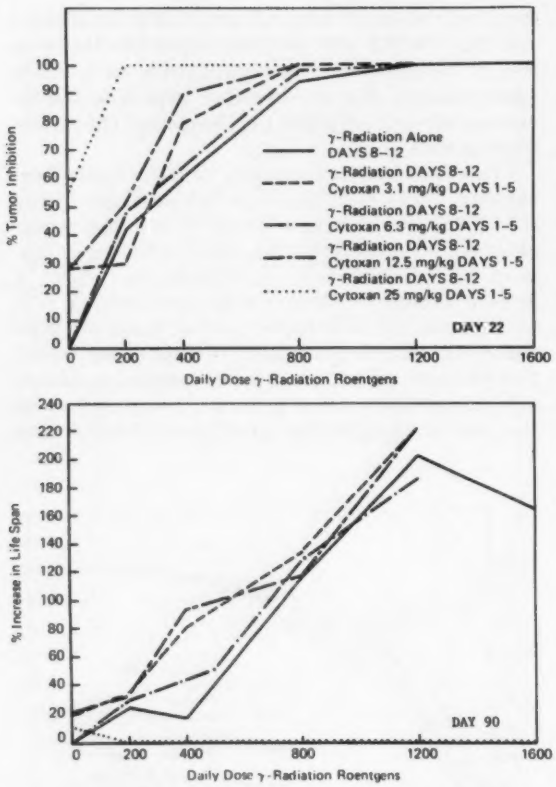
% Tumor Inhibition
γ-Radiation Alone DAYS 8–12
γ-Radiation DAYS 8-12 Cytoxan 3.1 mg/kg DAYS 1-5
γ-Radiation DAYS 8-12 Cytoxan 6.3 mg/kg DAYS 1–5
γ-Radiation DAYS 8-12 Cytoxan 12.5 mg/kg DAYS 1-5
γ-Radiation DAYS 8-12 Cytoxan 25 mg/kg DAYS 1-5
DAY 22
Daily Dose γ-Radiation Roentgens
% Increase in Life Span
DAY 90
Daily Dose γ-Radiation Roentgens

FIGURE 10.—Combined modality studies of γ-radiation and cyclophosphamide: effect on tumor growth and lifespan.

single agent which increased up to a maximum of 80% with doses of γ-radiation. The optimal ILS was 102% with 12.5 mg/kg of cyclophosphamide and 1200 R of γ-radiation which was lower than the values seen in the previous experiments (fig 14).

These last two treatment schedules have been repeated and the results confirm the findings that γ-radiation given on Days 8-12 prior to cyclophosphamide (Days 15-19) produces a longer survival and higher tumor inhibition than the obverse.

DISCUSSION

These studies showed that fractionated doses of γ-radiation were as effective as a single dose without producing local tissue damage or necrosis and that treatment of the local tumor early in the disease (Days 1-5) when the tumor weighed < 100 mg resulted in complete inhibition and increased lifespan. It was also possible to treat tumors weighing approximately 750 mg starting on Day 8 and to produce complete inhibition of the local mass. However, mice eventually died from metastases. The im B16 melanoma was very sensitive to γ-radiation and hence it was difficult to show any differential effects between γ-radiation alone and γ-radiation combined with 5-FU or cyclophosphamide.

Cyclophosphamide as a single agent was not too effective against the im implanted B16 melanoma and when combined with γ-radiation that was administered on Days 15-19 or given in combination to animals bearing 3-4-g tumors, there was only a 55%-72% inhibition of tumor growth with increases in host survival from 65% to 102%. The state of the tumor cells at this stage (possibly more hypoxic) may account for the lower sensitivity. Since the inhibition was not complete and the %ILSs were lower with γ-radiation alone, the delayed treatment schedule could serve as a useful model to explore other drug/γ-radiation combinations.

We have reported previously and in these studies (fig 2) that there was an inverse relationship between host survival and tumor-cell inoculum and this implied that the in vivo host survival system

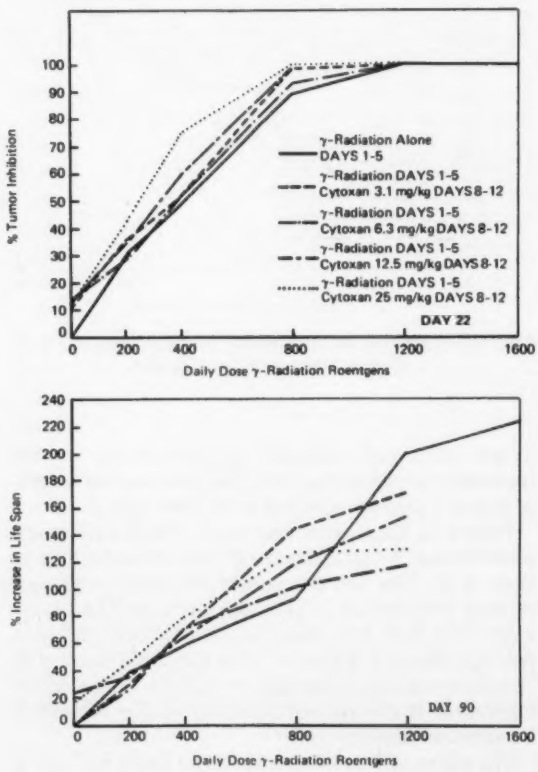
% Tumor Inhibition
γ-Radiation Alone DAYS 1-5
γ-Radiation DAYS 1-5 Cytoxan 3.1 mg/kg DAYS 8-12
γ-Radiation DAYS 1-5 Cytoxan 6.3 mg/kg DAYS 8-12
γ-Radiation DAYS 1-5 Cytoxan 12.5 mg/kg DAYS 8-12
γ-Radiation DAYS 1-5 Cytoxan 25 mg/kg DAYS 8-12
DAY 22
Daily Dose γ-Radiation Roentgens
% Increase in Life Span
DAY 90
Daily Dose γ-Radiation Roentgens

FIGURE 11.—Combined modality studies of γ-radiation and cyclophosphamide: effect on tumor growth and lifespan.

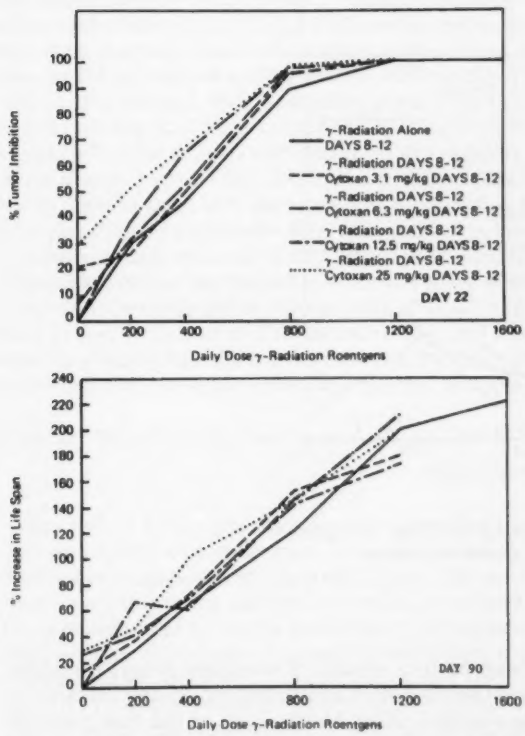

FIGURE 12.—Combined modality studies of γ-radiation and cyclophosphamide: effect on tumor growth and lifespan.

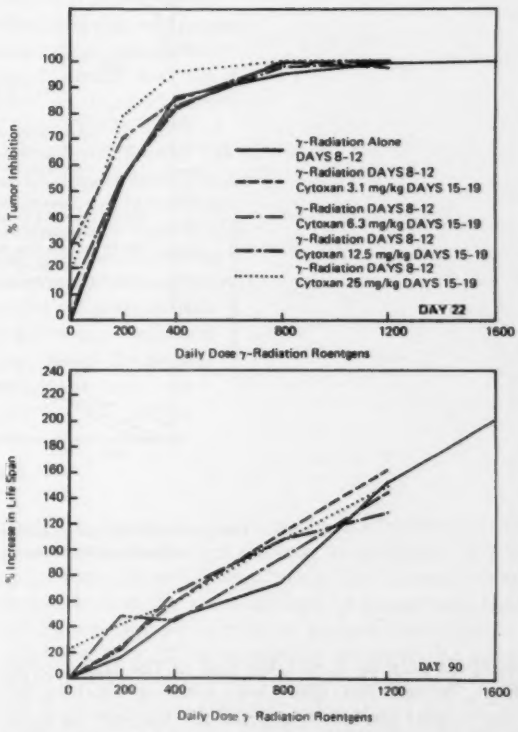

FIGURE 13.—Combined modality studies of γ-radiation and cyclophosphamide: effect on tumor growth and lifespan.

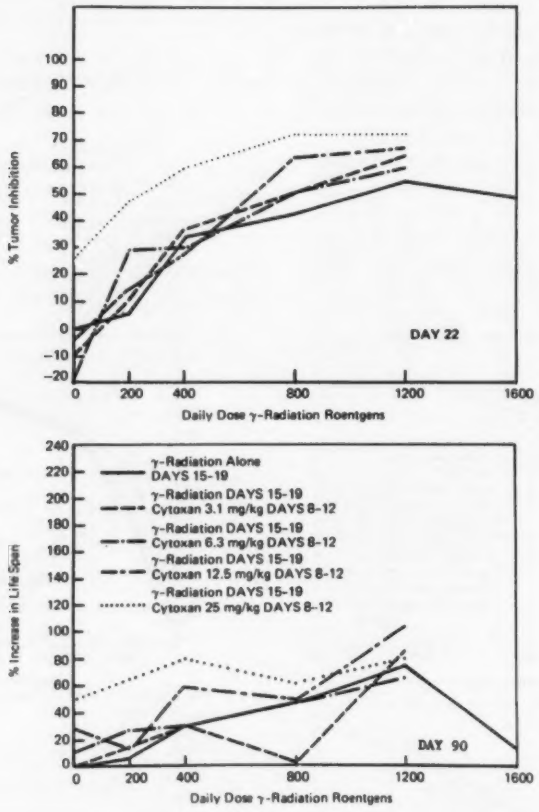
% Tumor Inhibition
DAY 22
Daily Dose γ-Radiation Roentgens
% Increase in Life Span
γ-Radiation Alone DAYS 15-19
γ-Radiation DAYS 15-19 Cytoxan 3.1 mg/kg DAYS 8-12
γ-Radiation DAYS 15-19 Cytoxan 6.3 mg/kg DAYS 8-12
γ-Radiation DAYS 15-19 Cytoxan 12.5 mg/kg DAYS 8-12
γ-Radiation DAYS 15-19 Cytoxan 25 mg/kg DAYS 8-12
DAY 90
Daily Dose γ-Radiation Roentgens

FIGURE 14.—Combined modality studies of γ-radiation and cyclophosphamide: effect on tumor growth and lifespan.

might be used as a reliable test of the therapeutic effect. In addition, there was some correlation between tumor inhibition and ILS for the various studies. However, the methods of tumor size and animal survival utilized in these studies may be too crude or insensitive to discriminate between the different therapy schedules, and the question as to whether these in vivo results of combined drug/γ-radiation treatments reflect additional cell killing remains to be clarified. Other assays such as the time required after complete regression of the B16 melanoma to regrow to a specific size or the number of metastatic lung colonies at a standard interval may be more discriminating and should be explored.

Combined Modality Therapy: Actinomycin D (NSC-3053) and γ-Radiation Against the Ridgway Osteogenic Sarcoma[1,2]

Philip C. Merker,[3] Isidore Wodinsky,[3] John M. Venditti,[4] and Joseph Swiniarski[3,5,6]

SUMMARY

The Ridgway osteogenic sarcoma (ROS), growing as an intramuscular (im) implant in $AKD2F_1$ mice, was used to study the antitumor activity of actinomycin D and ^{60}Co-γ-radiation in a combined modality experiment. Data showed that actinomycin D alone in the dose range of 0.025-0.075 mg/kg given intraperitoneally (ip) on 5 successive days, starting 45 days after tumor implantation, inhibited tumor growth. The tumors did not regress and the median survival times of treated animals were not significantly prolonged. ^{60}Co-γ-radiation given alone to the tumor-bearing leg in doses of 800 rads/day for 5 successive days, starting 38 days after tumor implantation, cured 90% of the animals with tumor. At 600 rads/day of γ-radiation exposure, 100% of the tumors regressed to 50% in size within about 7 days, with tumors barely palpable by 3 weeks and a 30% cure rate. The combination of 600 rads/day of γ-radiation given locally on 5 successive days, followed 3 days later by actinomycin D given ip for 5 successive days at a dose of 0.075 mg/kg/day, caused well-established im ROS to regress in 100% of the treated animals and cured 80% of the animals. Actinomycin D, in combination with 400 or 200 rads/day of ^{60}Co-γ-radiation, did not cure animals with tumor. The patterns of tumor regression and regrowth following single and combined modalities of treatment showed that this solid tumor model can be used in combined modality experiments designed for multiple courses of therapy.

[Cancer Chemother Rep Part 2, vol 5:225-233, 1975]

The use of drugs has significantly improved the control of solid cancer in man. However, it has recently been suggested by Zubrod (1) that the most likely avenue for the successful control of clinical cancers not cured by single modalities of treatment will be the combined use of surgery, x-radiation, and chemotherapy. However, Carter[7] has stated that a serious problem prevails in the allocation of clinical resources for the development of optimal combined modality strategy in the treatment of advanced disease states of different tumors. Accordingly, it is of prime importance to have "predictive" animal tumor models to uncover combinations of treatments that will have a high probability of success in the clinic.

Schabel et al (2) have recently reviewed the suitability of a number of solid transplantable tumors for use in experimental chemotherapy. One of the recommended tumors was the Ridgway osteogenic sarcoma (ROS), which was recommended primarily because it was the only solid animal tumor that regressed under treatment with vincristine, adriamycin, or actinomycin D. The ROS had also been recommended by Schmid et al (3) and Tarnowski et al (4) as one of eight tumors in a tumor spectrum for chemotherapy studies because it has suitable biologic characteristics and selective response to chemotherapy.

A number of investigators have reported that actinomycin D is especially effective against the transplantable ROS when administered as a single agent (3,4-10). Schwartz et al (9) showed that the growth of the ROS could be significantly inhibited after a single intravenous (iv) injection of the drug, and they

[1]Supported by contract N01-CM-33727 from Drug Research and Development Program (DR&DP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[2]Actinomycin D was supplied by DR&DP, DCT, NCI.

[3]Arthur D. Little, Inc., Cambridge, Mass.

[4]Drug Evaluation Branch, DR&DP, DCT, NCI, Bethesda, Md.

[5]The authors thank Dr. Frank M. Schabel at Southern Research Institute for his helpful suggestions during the course of this work.

[6]*Reprint requests to:* Dr. Philip C. Merker, Arthur D. Little, Inc., Acorn Park, Cambridge, Mass 02140.

[7]Carter SK. Report of the Associate Director, Cancer Therapy Evaluation (CTE). *In* Report of the Division of Cancer Treatment, NCI, 1974. Bethesda, Md, NCI, 1974, vol 2, pp 6.1-6.11.

proposed that the response of the tumor occurred without the assistance of immunologic mechanisms. The susceptibility of the ROS to actinomycin D was partially ascribed by Schwartz et al (9) to the innate instability of the tumor because (*a*) untreated tumors were found to consist of large regions of necrosis; (*b*) much of the proliferative activity of the tumor was described as being expended in the production of cells that did not survive; and (*c*) the variable population of tumor cells was possibly in a continuous state of high proliferative activity. Cell-cycle kinetic data recently reported by Miller and Simpson-Herren (11) showed that the viable portion of the tumor has a high proliferative fraction which persists throughout the growth of the tumor. A high rate of cell loss and the unique responsiveness of the tumor to drug therapy were attributed to these kinetic characteristics. Schwartz (10) reported that actinomycin D binds equally well to DNA obtained from either the ROS or from an actinomycin D-resistant 7,12-dimethylbenz(a)anthracene tumor; thus, the antitumor activity of the drug was not associated with special affinity to the DNA of the ROS.

Maddock et al (12) first reported that the ROS was sensitive to 400 rads of x-radiation given for three doses starting about 25 days after tumor implantation. In subsequent studies, D'Angio et al (5) showed that the tumor responded to treatment with the combination of actinomycin D and x-radiation, and the response of the tumor was found to be independent of the order in which the two treatments were given within a 24-hour period. The effectiveness of these combined modalities of therapy against the ROS is of importance in suggesting that the ROS may be a useful solid tumor model in studies that are directed towards finding appropriate combined modalities of treatment for clinical cancer. Therefore, a pilot experiment was done with actinomycin D and γ-radiation for the purposes of evaluating the utility of the ROS in combined modality experiments.

METHODS

The ROS, which was obtained from the Southern Research Institute, Birmingham, Alabama, is maintained in male or female AKR mice as a subcutaneous (sc) implant using tumor fragments inoculated by trocar. For experiments, tumor tissue from carrier mice was pooled in a petri dish kept on an ice bath. Approximately 5-10 g of tumor was added to 10 cc of Earle's salt solution and was lightly homogenized with a Potter Elvehjem homogenizer fitted with a motor-driven teflon pestle. One or two passes of the motor-driven pestle was used to prepare the tumor suspension. The tumor suspension was then diluted 1:5 (wt/vol) with Earle's salt solution, and 0.25 cc was injected into the muscle of the right hind leg of $AKD2F_1$ male or female mice weighing about 20-25 g. Tumor size was determined with vernier calipers by measuring the tumor diameters and then calculating the area in square millimeters.

Groups of ten tumor-bearing mice were used for each dose level of treatment. Mice were kept in suspended cages with mesh floors. Food and water were given ad libitum.

^{60}Co-γ-radiation treatment was provided by a Tech/Ops Model 624 Gamma Ray Irradiator. This is a remotely operated self-contained instrument, capable of providing a delivered dose which is isodose (± 10%) over any 2-inch high horizontal disc region within the 6-inch high, 14-inch diameter chamber.

The source is located 22 inches from the center of the 60-inch high irradiation chamber; a source intensity corrector plate is incorporated in the irradiator to compensate for lateral variations in dose rate. Because the source and chamber are coaxial, the intensity distribution is axially symmetric.

γ-Radiation treatment was given only to the tumor-implanted leg at a dose rate of 90 rads/minute, and the dose rate was determined with a Bendix Dosimeter using an exposure time of 5 minutes. The whole body, with the exception of the leg, was shielded with 2 inches of lead and the whole-body exposure through the shielding was calculated to be about 5% of the administered dose. During γ-radiation, mice were anesthetized with sodium pentobarbital (Nembutal) at an average dose of 70 mg/kg intraperitoneally (ip). Doses of radiation and details of scheduling of treatment are given in the figures and tables.

Actinomycin D was prepared immediately before use in 0.86% aqueous NaCl solution; each daily dose was given ip in a final volume of 0.5 ml. The doses and schedules of treatment, in combination with γ-radiation, are given in the figures and tables.

EVALUATION OF RESULTS

^{60}Co-γ-Radiation Therapy Alone

Single dose (fig 1).—At 800 rads tumor growth was inhibited but regression did not occur, and at 1200 rads tumors regressed slightly. Following exposure to 1600 or 2000 rads, tumors regressed to about 50% of initial size. The nadir occurred at about 10-15 days and thereafter the tumors rapidly regrew. The median survival time (MST) was modestly increased by 39% at the 1600- and 2000-rad doses. The MST of mice that received 2400 rads was less than that of controls suggesting that the γ-radiation was lethal.

Fractionated doses (figs 2 and 3).—Fractionated doses of γ-radiation of 800 rads/day, administered on

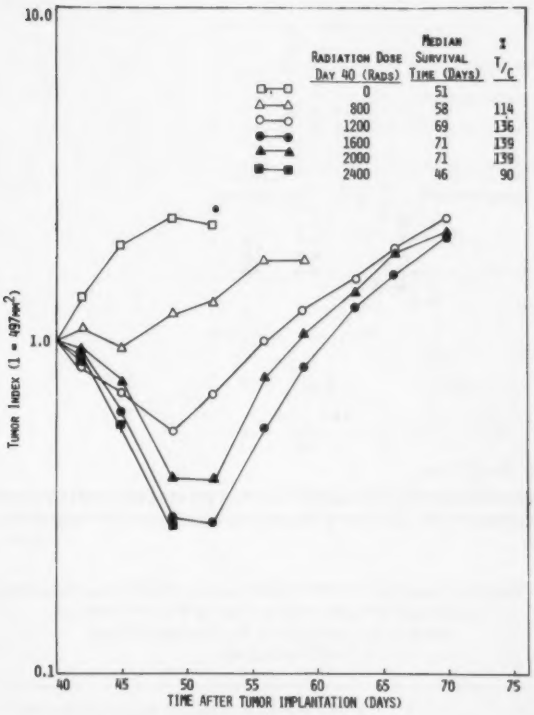

FIGURE 1.—Effect of a single dose of ^{60}Co-γ-radiation on the growth of im ROS in AKD2F$_1$ mice. T = MST (days) of treated animals (10 animals per group); C = MST (days)of untreated control animals (10 animals per group). Tumor index = average tumor area (mm²) after therapy divided by average tumor area (mm²) when γ-radiation was started on Day 40. γ-Radiation was given to the tumor-implanted leg only; whole-body shielding, 2-inch lead; dose given at an average rate of 90 rads/minute. For additional details of radiation see Methods section. % T/C = T/C × 100.

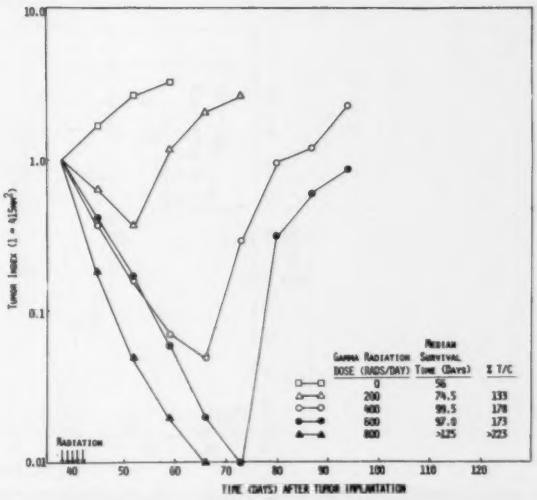

FIGURE 2.—Effects of fractionated doses of ^{60}Co-γ-radiation on the growth of ROS in AKD2F$_1$ mice. T = MST (days) of treated mice (10 animals per group); C = MST (days) of untreated control mice (10 animals per group). Tumor index = average tumor area (mm²) after therapy divided by average tumor area (mm²) when γ-radiation was started on Day 38. γ-Radiation was given to the tumor-implanted leg only; whole-body shielding, 2-inch lead; dose given at an average rate of 90 rads/minute. For additional details of γ-radiation see Methods section.

5 successive days, caused well-established ROS to regress in size to barely palpable tumors within 3-4 weeks after treatment (fig 2). Tumors treated with 600 rads/day regressed in all animals to barely palpable size and reappeared in 70% of the treated animals. They grew to their original size within about 3 weeks from the nadir, and the MST increased by 73%. The 400-rads/day dose of γ-radiation also caused tumors to regress; the nadir was approximately 4 weeks after the last γ-radiation treatment. Thereafter, within 3 weeks after the nadir tumors regrew to their original size in all animals. The MST of animals treated with 400 rads/day was moderately prolonged by 78% in contrast to untreated animals. About 50% of the tumors treated with 200 rads/day decreased in size.The tumors then rapidly regrew, and the MST was increased by only 33% in relation to untreated animals. The percentage survival curves shown in figure 3, for animals treated for 5 successive days with 200, 400, and 600 rads/day of γ-radiation, were shifted to the right and parallel to the curve for untreated animals. It should be noted that the 50% survival times for the dose levels of 600 and 400 rads/day were essentially similar, but all of the animals treated with 400 rads/day were dead by the 110th day whereas 30% of the animals treated with 600 rads/day survived beyond this time. Data for percentage survivors show the "curative" action of fractionated doses of γ-radiation: 90% of the animals that were treated with 800 rads/day and 30% that were treated with 600 rads/dav were judged to be tumor free at 80+ days after the last γ-radiation treatment.

Actinomycin D Therapy Alone

Actinomycin D therapy was given ip as a single daily dose for 5 successive days starting on the 45th day after tumor transplantation. Tumor growth was inhibited by actinomycin D during the course of treatment in the dose range of 0.025-0.075 mg/kg, but inhibited tumors did not regress. Three days after the last dose of drug, the tumors rapidly regrew. The MST of animals treated at 0.025 mg/kg was 60 days or only 4 days longer than control animals, and it was 52 days at the 0.075-mg/kg dose level of drug or about 4 days less than controls.

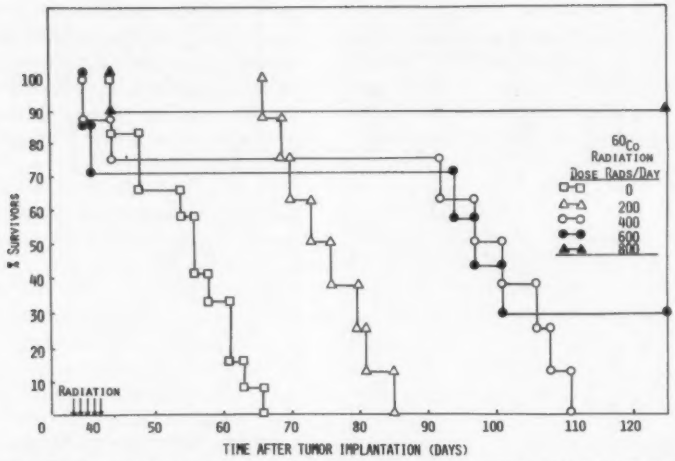

FIGURE 3.—Percentage of surviving animals after localized ^{60}Co-γ-radiation treatments to well-established ROS growing im in AKD2F$_1$ mice. γ-Radiation was given to the tumor-implanted leg only; whole-body shielding, 2-inch lead; dose given at an average rate of 90 rads/minute. Ten tumor-bearing animals per group at start of therapy.

Combined Modality Experiments

In these experiments γ-radiation treatment, which were started 38 days after tumor implantation, were given for 5 successive daily doses of 200-800 rads/day. Actinomycin D therapy was started 3 days after the last γ-radiation treatment using the range of doses of 0.013-0.075 mg/kg given for 5 successive days.

MST of treated (T) versus control (C) groups (%T/C, table 1).—Data show that actinomycin D in doses of 0.025 and 0.075 mg/kg, in combination with 600 rads/day of γ-radiation therapy, increased the MST of treated animals over and above that obtained when either treatment was given separately. The total amount of γ-radiation was reduced from 4000 to 3000 rads when actinomycin D was combined in the treatment plan, but this combination was not found to be superior to 800 rads/day given alone. Actinomycin D, in combination with 400 rads/day, was somewhat more effective than 400 rads/day given alone. The combination of 800 rads/day and actinomycin D at 0.075 mg/kg was found to be toxic.

Percentage survival data (fig 4).—Data for 600 rads/day of γ-radiation, in combination with the 0.075- and 0.025-mg/kg doses of actinomycin D, showed that 50%-80% of the tumor-bearing animals were alive 80+ days after the end of therapy. In contrast, < 50% of the animals in the other treatment groups were alive at the same time period.

Percentage responders (fig 5).—Animals that showed at least a 50% reduction in tumor size after therapy with no further progression in tumor growth were classified as responders. Data for the combination of 0.075 mg/kg of actinomycin D and 600 rads/day of γ-radiation treatment show that over 90% of the animals were classified as responders throughout the course of the experiment. Over 60% of the animals treated with the combination of 0.025 mg/kg of actinomycin D and 600 rads/day of γ-radiation were classified as responders more than 70 days after the last dose of drug had been given.

Tumor size measurements (figs 6 and 7, table 2).—Tumor-bearing animals treated with γ-radiation alone (600 rads/day) and in combination with actinomycin D showed that tumors rapidly regressed during and after the course of therapy. Figure 6 shows

TABLE 1.—Response (% T/C) of ROS-bearing AKD2F$_1$ mice following treatment with actinomycin D alone, ^{60}Co-γ-radiation alone, or the combination of actinomycin D and γ-radiation*

Actinomycin D (mg/kg/day)	%T/C† at ^{60}Co-γ-radiation dose (rads/day) of—				
	0	200	400	600	800
0	0	133	178	173	>223
0.013	104	141	192	206	>223
0.025	107	143	182	>223	>223
0.050	93	158	193	207	>223
0.075	93	168	191	>223	84

*Radiation was given to tumor-bearing leg only; 5 daily treatments starting on the 38th day after tumor implantation. Drug was given ip as a single injection for 5 days starting 3 days after the last γ-radiation treatment when tumors were 45 days postimplantation.

†T = MST (days) of treated animals; C = MST (days) of control animals. Ten animals per treatment group.

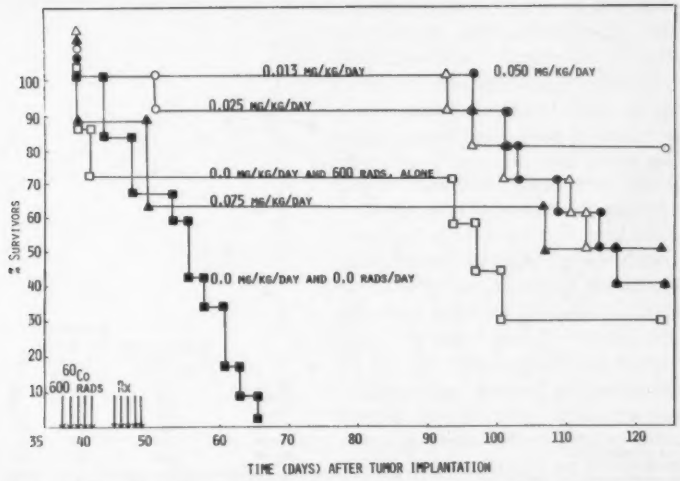

FIGURE 4.—Percentage of surviving ROS-implanted animals after localized ^{60}Co-γ-radiation (600 rads/day) given alone and in combination with actinomycin D. Treatment plan as described in Methods section and table 1. Ten tumor-bearing animals per group at start of experiment.

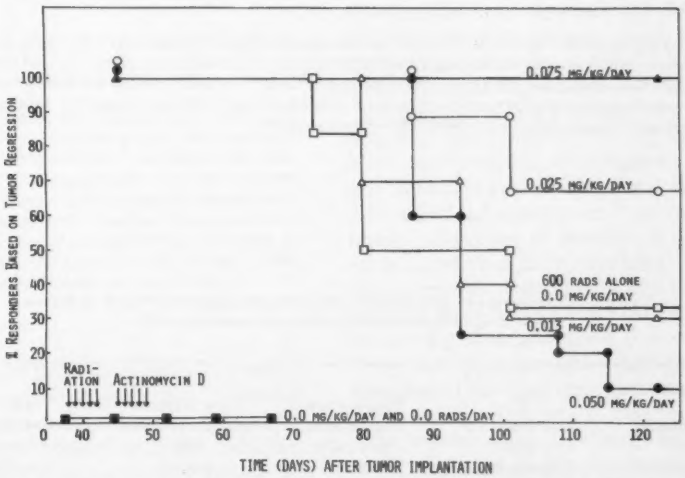

FIGURE 5.—Percentage of ROS-implanted animals responding to therapy with ^{60}Co-γ-radiation (600 rads/day) given alone and in combination with actinomycin D. Treatment plan as described in Methods section and table 1. Ten animals per treatment group. Responders = tumor regression of 50% with no further progression in growth.

that the times for tumors to regress by 50% (6-9 days) were similar for all groups of treated animals, but differences were noted among the times it took for tumors to regrow to about the size when treatment was started (table 2). Data for the therapy schedule of 600 rads/day plus actinomycin D show that the time for tumor regrowth was related to subsequent MST, ie, the longer it took for tumors to regrow to original size the longer the animals survived, and when tumors grew back to their original size, animals died within 1-2 days (table 2). Tumor size measurement data for the combination of 200 rads/day alone and in combination with actinomycin D are shown to illustrate the extent to which the tumor was inhibited by this combined treatment (fig 7). Data show that γ-radiation at a dose of 200 rads/day plus actinomycin D at 0.050 or 0.075 mg/kg maximally inhibited tumor growth. Tumor regression occurred 10-15 days following the last actinomycin D injection, but tumors in all groups rapidly grew to

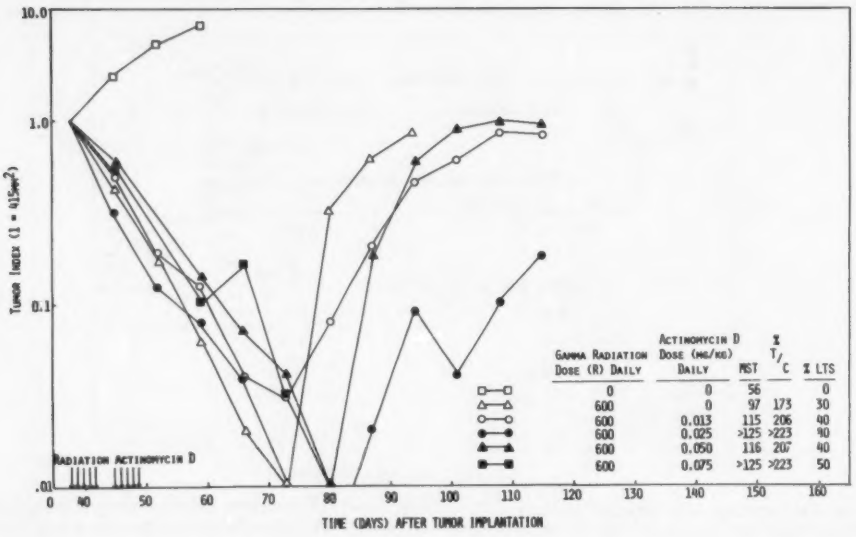

FIGURE 6.—Effect of ^{60}Co-γ-radiation (600 rads/day) given alone and in combination with actinomycin D on the growth and regression of im ROS in $AKD2F_1$ mice. Tumor index = average tumor area (mm^2) after therapy (10 animals per group) divided by average tumor area (mm^2) at time therapy was started (10 animals per group); % T/C = MST of treated (T) animals divided by MST of control (C) animals × 100; % LTS = pefcent long-term survivors calculated from the number of animals living ≥ 120 days divided by total number of animals in group (10 per group) × 100. Treatment plan as described in Methods section and table 1.

TABLE 2.—ROS regression and regrowth following therapy with ^{60}Co- γ-radiation given alone and in combination with actinomycin D*

γ-Radiation dose (rads/day)	Actinomycin D dose (mg/kg/day)	Time for 50% reduction in tumor size (days)	Time to return to original tumor size (days)	MST (days) from start of treatment (Day 38)
0	0	—	—	16
600	0	6	57	59
600	0.013	7	70	77
600	0.025	9	>82	>82
600	0.050	9	77	78
600	0.075	7	>82	>82

*Tumor size was determined as tumor area (mm^2). Start of γ-radiation, 38 days after tumor implantation; start of actinomycin D treatments, 45 days after tumor implantation. Treatment plan as described in Methods and table 1.

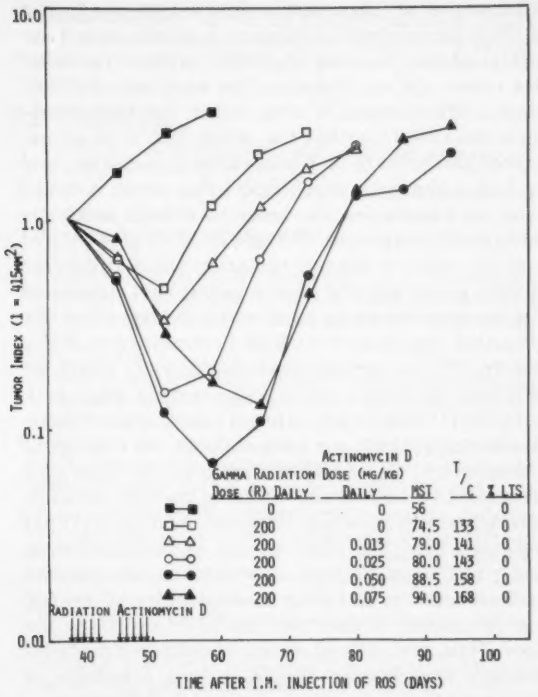

FIGURE 7.—Effect of ^{60}Co-γ-radiation (200 rads/day) given alone and in combination with actinomycin D on the growth and regression of im ROS in AKD2F$_1$ mice. Tumor index = average tumor area (mm²) after therapy divided by average tumor area (mm²) at start of therapy; % T/C = MST of treated (T) animals divided by MST of control (C) animals × 100. % LTS = percent long-term survivors calculated from the number of animals living ≥ 120 days divided by total number of animals in group (10 per group) × 100. Treatment plan as described in Methods section and table 1.

pretreatment size within 10-20 days after the nadir. The MSTs of animals treated with this combined therapy regimen were not significantly prolonged over the MSTs of animals treated with 200 rads/day alone.

DISCUSSION

The experimental transplantable animal tumors currently used for routine primary screening of drugs and chemicals by the Drug Evaluation Branch, DR&DP, DCT, NCI, are: L1210 lymphoid leukemia, B16 melanocarcinoma, and P388 lymphoblastic leukemia. Other transplantable tumors are available, including ROS.[8]

[8]Interim change to Instruction 14, Nov 1973. Screening data summary interpretation and outline of current screening. Prepared by Drug Evaluation Branch, DR&DP, DCT, NCI. (RO has been selected as the standard abbreviation for use by screeners.)

Recently, Schabel et al (2) drew attention to the unique responsiveness of the ROS to actinomycin D, vincristine, and adriamycin, and they suggested that this tumor be used as an advanced solid tumor model in chemotherapy studies because of its responsiveness to these three drugs. In addition, the responses of the tumor to treatment can be "staged" in terms of partial and complete regression, duration of remission, and "cure" rates.

Goldin and Johnson (13) recently reviewed the activity of actinomycin D against animal tumors and categorized the ROS as one of two tumors that could be cured by actinomycin D treatment. When the ROS was first used by Sugiura (7) to test actinomycin D, the drug was found to cause complete regression in 96 out of 120 established tumors (80% cured). In studies that showed actinomycin D to be highly effective against the ROS, therapy was started at varying times after transplantation, ie, 5-7 days (4,7,8), 12 days (2,6), or 20-21 days (5,9). The tumor has been used in chemotherapy experiments as an sc (2,4,7-9) or intramuscular (im) (5) implant. Effective doses and schedules and routes of administration for actinomycin D, when it was used as a single chemotherapeutic drug against the ROS, have been reported to be: 0.6 mg/kg ip, single dose (2); 0.8-1.2 mg/kg iv, single dose (9); and 0.025-0.05 mg/kg ip, given in seven successive daily doses (7,8).

In our studies, the ROS was implanted im and therapy with actinomycin D alone was started 45 days after implantation when tumors were large (about 415 mm²) and animals only had about 10 days of additional life. Actinomycin D therapy, which was given ip in the range of doses from 0.025 to 0.075 mg/kg on 5 successive days, inhibited tumor growth, but the tumors did not regress and the MSTs of treated animals were not significantly prolonged. The lack of activity of actinomycin D alone against the ROS under our experimental conditions can probably be ascribed to the fact that systemic therapy with this drug was started late in the course of the disease when tumors were quite large and growing progressively. Schabel (6) has shown that large 18-day-old progressively growing sc implants of ROS were less responsive than small 12-day-old sc implants to the action of actinomycin D, adriamycin, 5-fluorouracil, and melphalan. Although systemic treatments of actinomycin D alone did not cause ROS to regress or cure animals under our experimental conditions, ^{60}Co-γ-radiation given alone to the tumor-bearing leg in fractional doses of 800 rads/day for 5 successive days cured animals of tumor.

The combination of 600 rads/day of ^{60}Co-γ-radiation given locally to the leg on 5 successive days followed 3 days later by systemic treatments with actinomycin D, given for 5 successive days in doses of

0.075 mg/kg, caused well-established im ROS to regress in 100% of the treated animals; 80% of the animals treated with this combination of therapy were "cured" of the disease. Therefore, our data support the findings of Maddock et al (12) and D' Angio et al (5) that the ROS is radiosensitive and the combination of γ-radiation and actinomycin D is an effective combined modality of treatment against ROS.

Data for tumor growth and regression showed that the γ-radiation dose of 600 rads/day caused a greater percentage of the tumors to regress to barely palpable size than did the 400- and 200-rads/day doses of γ-radiation. Also, the length of time for tumor to regrow back to original size was longer in animals treated with 600 rads/day than in animals treated with the other two doses of γ-radiation. Therefore, it is highly probable that regressing tumors treated with 600 rads/day of γ-radiation had fewer numbers of remaining viable cells than regressing tumors treated with 400 or 200 rads/day of γ-radiation. Under these circumstances, it would be expected that actinomycin D would be more effective against regressing tumors that had been treated with 600 rads/day of γ-radiation because these tumors probably contained the smallest number of viable cells. Additional experimentation is, however, required to determine quantitatively the number of viable cells contained in regressing tumors that have been treated with various dose levels of γ-radiation alone and γ-radiation combined with actinomycin D, as has been done by Wodinsky et al (14) for the L1210 leukemia.

The interval between the times when tumors regressed to 50% of their size and then regrew to their original size following combined modality therapy was found to be sufficiently long to allow for additional courses of treatment. This was also noted by D'Angio et al (5), and they found that a second course of combined modality therapy resulted in a higher percentage of cures. Schabel (6) has also shown that this tumor model can be used in experiments designed for repeated courses of therapy with single drugs or sequential courses of two drugs. Therefore, the solid transplantable ROS model can be used to simulate the clinical situation in which therapeutic intervention consists of a number of courses of treatment that are given to induce and then maintain remission of solid cancers. This would be similar to the use of the spontaneous AKR lymphoma in a two-stage therapeutic experimental design meant to simulate clinical trials of leukemia and lymphoma (15).

Combined modality experiments can be designed in a number of ways: drugs can be administered prior to, at the same time as, or following γ-radiation. Wodinsky et al (16) recently showed that the timing of drug administration relative to whole-body γ-radiation of mice bearing the P388 lymphocytic leukemia tumor did not influence the maximum antineoplastic effectiveness of nine drugs; the only exceptions were mechlorethamine, which had to be administered just prior to or 4 hours after γ-radiation, and cyclophosphamide, which had to be given 4 hours prior to γ-radiation, in order to obtain maximum therapeutic responses. D'Angio et al (5) also showed that the order in which γ-radiation and actinomycin D were given and the time interval that separated the two treatments up to 24 hours did not affect the enhanced response of the ROS to combined modality therapy. In the current combined therapy study, an enhanced antitumor effect was obtained when actinomycin D therapy was started 3 days after γ-radiation therapy; thus, our data support the findings of D'Angio et al (5) and Wodinsky et al (16) that each treatment in a combined modality therapy schedule can be given separately. However, Andrews (17) has indicated that the effectiveness of combined drug and γ-radiation therapy depended on the relative radiosensitivity and drug susceptibility of normal and neoplastic tissues and the kinetics of the tissues. Thus, the optimal design of combined modality therapy experiments should include schedules of treatment that take into account such differences.

The current experiments have demonstrated the potential usefulness of the advanced ROS model in combined modality therapeutic studies. Our overall findings substantiate the conclusions of Schabel et al (2) that the ROS should provide data of practical value to clinical cancer chemotherapy. The patterns of response of this advanced solid tumor model to the combination of ^{60}Co-γ-radiation and actinomycin D indicated that the tumor model can be used in an experimental design that includes multiple courses of therapy involving a number of modalities of therapy. In this regard Hahn et al (18) have shown that local hyperthermia to established ROS consistently increased the antitumor effects of actinomycin D. Therefore, the ROS can be a potentially useful solid tumor model in experiments designed to uncover new combined modalities for the therapy of clinical cancer.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053
Adriamycin: NSC-123127; CAS reg. No. 23214-92-8
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan
5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8
Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-; L-sarcolysin
Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Interaction of Experimental and Clinical Studies in Combined Modality Treatment

Stephen K. Carter and Todd H. Wasserman[1,2]

The main responsibility of the Division of Cancer Treatment (DCT) of the National Cancer Institute is research in the treatment of cancer, with the long-term objective of curing or completely controlling cancer in man. Rather than a single disease, cancer includes over 100 related but unique forms of a disease in which uncontrolled proliferation of abnormal cells without therapeutic intervention results in death of the patient. Each disease requires a strategic use of all the available therapeutic modalities modulated to meet the exigencies of the natural history of the tumor.

In general, cancer can be separated into two major classifications—solid tumors and hematologic malignancies. Solid tumors originate in a specific tissue or organ site and can be eradicated by local modalities of therapy if diagnosed at an early stage. These tumors are the major cause of death from cancer and include the big three among the killers, ie, lung cancer, breast cancer, and large bowel cancer. The hematologic malignancies are disseminated at the time of diagnosis in nearly every case and local therapy cannot be utilized.

Two forms of local therapy exist—surgical treatment and radiotherapy. Surgical treatment is the modality that cures the greatest number of cancer patients today. Progress in cancer surgery is responsible for extending survival for 5 years, but not necessarily achieving cure, in at least 85% of patients with skin cancer, in about 60% of women with breast cancer, in about 40% of patients with large bowel cancer, and in 70% of women with cancer of the uterus.

Radiotherapy can be highly effective in certain tumors where surgical treatment is not possible, such as cases where cancer cells are at a site inaccessible to surgical removal. In addition, there are a few tumors where irradiation can achieve results comparable or superior to radical surgical excision. In patients whose disease is detected early, radiotherapy has successfully extended survival for 5 years or more in at least 90% of men with seminoma of the testis, in at least 80% of children with retinoblastoma, in about 75% of patients with early Hodgkin's disease, and in about 50% of patients with squamous cell cancer of the cervix or of the nasopharynx.

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Basis of the Combined Modality Approach

Unfortunately, in a great number of patients the local therapeutic approaches fail to control the tumor. Each tumor type fails with a pattern unique to itself and predictable to some degree based on past observations. By identifying the first recurrence or reappearance of a tumor it is possible to attempt the design of a corrective therapeutic maneuver or strategy that will overcome the reason for failure.

Patients with malignant tumors fail to respond to initial therapy because it does not control the primary tumor (T), the nodal involvement (N), or dissemination to distant visceral sites (M). As Rubin et al (1) have pointed out, there are seven potential patterns of treatment failure involving T, N, or M alone or in some combination (fig 1). Glioblastoma multiforme is one example of a tumor where treatment fails because of the inability to ablate the local tumor. Nodal failures are a particular problem in squamous cell cancers of the head and neck area and in lung cancer. The great majority of patients who fail to respond to therapy are afflicted with disseminated disease and this is the most common route of failure in the major cancer killers. Obviously T, N, or M failures are combined in a large number of patients. It is probable that in tumors where the T and N compartments are the overt reason for failure, better local and regional control are bound to unmask metastatic disease.

A major approach to improving the cure rates in cancer treatment involves the integration of chemotherapy with the local modalities of surgical treatment and radiotherapy applied against solid tumors. Such an approach has been elucidated by the DCT (2). The philosophic base of this combined modality approach is the recognition that surgical treatment and radiotherapy are local modalities which kill tumor cells only where they are applied. They fail to cure many patients even when they remove all the tumor visible to the naked eye or on diagnostic roentgenograms. This failure is felt to be due to the presence of disseminated microscopic disease foci at the time of surgical excision of the primary tumor,

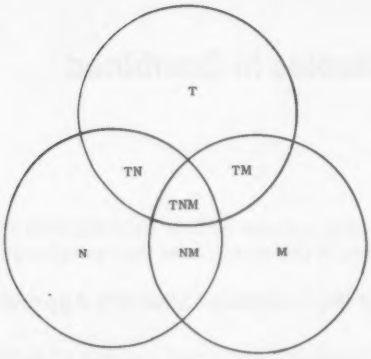

FIGURE 1.—Failure pathways for T (primary tumor), N (nodal involvement), and M (dissemination), and their combinations.

which often includes the surrounding tissue and part of the regional lymph nodes.

Chemotherapy, when used optimally, has the potential for eradicating the metastatic foci of early disease. In the DCT strategy, the drug regimens showing the highest degree of activity in advanced disease will be the prime candidates for use in the combined modality approach. The degree of cell kill necessary to shrink a bulky solid tumor mass by more than 50%, which is usually the minimum definition of objective regression, is quite large. If this level of cell kill could be directed against the relatively small tumor burden remaining after surgical excision, perhaps eradication of the last neoplastic cell could be achieved.

Experimentally, it is well established that the best chance of eradicating a tumor mass with chemotherapeutic agents is when the tumor cell population is small, a situation occurring immediately after resection of the primary tumor. The ability of chemotherapy to produce a cure is greater when all visible tumor is surgically excised than after subtotal resection (3-5). The inverse relation between tumor cell population and chemotherapeutic cure has been clearly expressed in quantitative terms in mice bearing the L1210 transplanted leukemia (6). Similar conclusions have been reached in experimental solid tumor systems (7).

The DCT-proposed therapeutic strategy for increasing cure rates in solid tumors involves integration of drugs into combined modalities for primary treatment according to the following sequential approach: (*a*) test new drugs and combinations in advanced disease; (*b*) develop an optimum chemotherapy regimen for primary treatment of disseminated disease; and (*c*) integrate this optimum chemotherapy regimen into a combined modality approach for primary treatment of local and regional disease. In this scheme, new drugs and drug combinations would be tested in advanced disease and those drugs that showed positive results would be used in the primary treatment of disseminated disease. The optimal regimen evolved in this situation would then be integrated into a combined modality approach for primary treatment of local and regional disease.

Such an approach in the therapeutic attack against breast cancer is schematically outlined in figure 2. The solid arrows show the standard treatment flow in this disease, beginning with mastectomy and moving through hormonal manipulation and eventually to chemotherapy. The broken arrows indicate the concerted efforts of the DCT, the Breast Cancer Task Force, and the Division of Cancer Research Resources and Centers that might integrate chemotherapy into the earlier stages of treatment.

Breast cancer is one area where combined modality therapy is achieving some preliminary positive results. In earlier studies, Nissen-Meyer et al (8) in Scandinavia and Fisher et al (9) reported improved survival in patients with operable breast cancer treated with short courses of chemotherapy. Nissen-Meyer and his co-workers observed improved survival at 6 years using only a short course of postoperative cyclophosphamide. Fisher et al noted a significant decrease in recurrence rates among premenopausal women with four or more positive nodes after a single course of thioTEPA. More recently, Fisher et al (10) reported that a 20% survival improvement demonstrated at 5 years has persisted at 10 years. Both of these studies were based on the concept that tumor cells are disseminated at the time of surgical excision and that drug treatment will prevent metastases.

Two recent studies illustrate how adjuvant chemotherapy, when applied for longer times, has significantly delayed disease recurrence. Fisher et al reported a study which included more than 250 patients at 37 hospitals (11). The study employed melphalan at a dose level of 0.15 mg/kg/day for 5 days postoperatively in patients with positive nodes after mastectomy. The data obtained after 2 years indicate that the recurrence rate has been reduced significantly in women receiving melphalan. The results are particularly striking in premenopausal women where tumor recurrence was observed in only one of 30 patients receiving the drug as compared with 11 recurrences among 37 patients who underwent surgical treatment alone. For postmenopausal women, recurrence rates were also reduced in those treated with melphalan, but not as markedly as in the premenopausal group.

The second study was performed in the National Cancer Institute of Milan (Italy) by Bonadonna et al (12) under contract to the DCT. Using the same

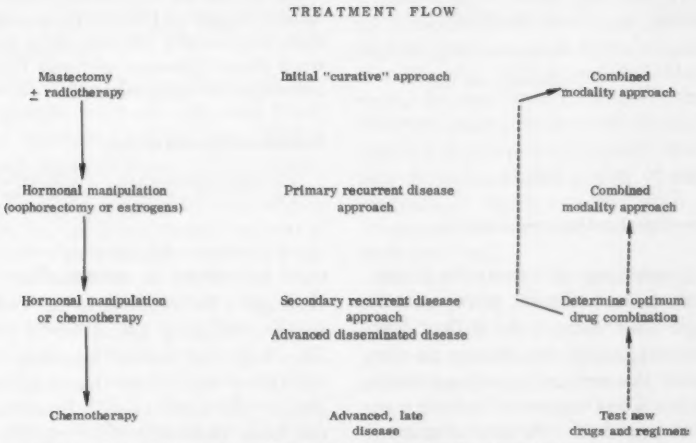

FIGURE 2.—Proposed integration of chemotherapy into combined-modality treatment of earlier stages of breast cancer.

stratification and eligibility criteria as the Fisher study, they allocated patients with positive nodes after mastectomy to receive either a three-drug combination called CMF (cyclophosphamide, methotrexate, 5-fluorouracil [5-FU]) or surgical treatment alone. After 18 months of patient accrual, 114 patients have been entered into the surgical treatment alone study arm and 87 into the CMF study arm. Fourteen patients (12%) have relapsed in the surgical treatment alone arm and one (1%) relapsed in the CMF plus surgical treatment regimen.

Interaction of Experimental and Clinical Studies

The clinician approaches the design of therapeutic trials with myriad possible choices. These choices are largely empirical, based on the clinician's rational analysis of past clinical experience and his feelings about what might have the best chance of success. He looks to the experimentalist for aid in making these choices and has at his disposal a vast amount of experimental data which have accumulated over many years of laboratory study. Unfortunately, in many cases the clinician and experimentalist work parallel to one another with only a few meager bridges of interaction that generally do not cross the clinical-experimental gap at the critical juncture.

Figure 3 illustrates the potential inputs into an experimental or clinical trial and the potential parallel and/or interactive aspects of the two systems. One of the critical areas in a combined modality strategy where the experimentalist and the clinician need to interact is in the combination of surgical treatment plus drugs.

Surgical Treatment and Drugs

Experimental screening for surgical adjuvant drugs can be approached in two ways. One approach is to use experimental systems to test for a potentially positive therapeutic interaction among a selected universe of drugs. The number of drugs that should be in this universe and their sources relate to where the clinicians are choosing drugs for human studies.

One approach to the question is to select drugs based purely on their postulated mechanism of action as supported by either experimental data or nonspecific clinical activity. Examples of such approaches being used today are in the field of immunotherapy where a range of materials such as bacillus Calmette-Guérin (BCG) and *Corynebacterium parvum* are being given in postsurgical situations based on their experimental activity and their ability to apparently enhance immunologic responsiveness in some patients. The clinical "activity" is nonspecific in that it is not disease oriented and, in many cases, these materials are being employed in adjuvant situations in the face of almost no previous clinical data for the specific tumor. Other examples that might occur in the future in a similar manner involve possible antimetastasis compounds and reverse transcriptase inhibiting drugs.

Another approach is to choose clinically active drugs that have shown the ability to kill tumor cells in advanced disease. Again, the question could be raised as to whether this activity should or should not be disease specific. Should investigators evaluate a drug postsurgically in breast cancer based on activity in lymphomas or malignant melanoma, or should activity against advanced breast cancer be required?

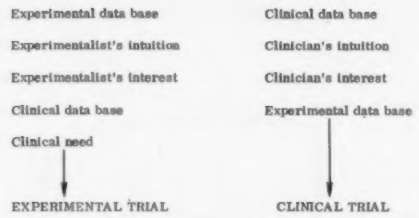

FIGURE 3.—Inputs to clinical and experimental trials.

In actuality, the combining of cytotoxic drugs with surgical treatment has almost always been along disease-oriented lines using a drug that has some degree of activity against the tumor in the advanced state. One of the critical questions that needs to be answered is what degree of activity in advanced disease is required of a drug or regimen before a significant gain in survival can be expected in the adjuvant situation where the drug is given in the hope of eradicating microscopic disease after surgical removal of the bulk of tumor. There are now a wide variety of adjuvant trials ongoing in breast cancer using a wide range of regimens involving chemotherapy and immunotherapy. When the activity of these regimens is examined in advanced disease, the activity ranges from zero (untested) for BCG, through 19% for melphalan, to over 50% for a wide range of combinations. Only time will tell what the correlations will be and whether the advanced-disease state is the place to screen for activity in a surgical adjuvant situation.

A second approach to screening in surgical treatment plus chemotherapy is to attempt to help the clinician use the compounds he has selected for high priority in this situation. With any given drug in an adjuvant situation there is a wide range of variables affecting their use. Among the most critical variables are the following:

Sequence.—Should the drugs be given before surgical excision, interoperatively, or postoperatively? Assuming postoperative use, which is the most common, how long an interval should elapse between surgical treatment and the initiation of the adjuvant study?

Schedule.—What schedule of drug administration should be used?

Dose level.—Should a full therapeutic dose level be administered or should this be lowered by some factor to avoid the risk of significant toxicity? What level of toxicity, if any, should be sought in the adjuvant situation?

Duration.—How long should the drug be administered? Should it be 1 or 2 years, or until recurrent disease occurs, all of which are options currently being clinically evaluated.

Clearly there are numerous ways for employing a single drug in a postsurgical situation and the clinician empirically chooses only one or two. This is an area where greater aid and input from the experimentalist is badly needed.

Radiotherapy and Drugs

The combination of radiotherapy and drugs, which can be clinically accomplished in a variety of ways, is another important area for interaction. One of the most common approaches is to use drugs to potentiate the effect of radiotherapy on localized tumor through the mechanism of radiosensitization. An ideal sensitizing agent would modify the radiation dose-response curve, allowing tumor control at a dose level well below the range of normal tissue tolerance. As Goffinet and Bagshaw (13) have stated, the ideal radiosensitizer would shift the tumor radiation-response curve away from that of normal tissue so that little or no normal tissue effect occurs when a tumoricidal dose of radiation is reached, if selective uptake of the radiosensitizer into tumor cells could be obtained.

Bleehen (14) has pointed out that potentiation of the radiation effect with increased cell killing may be associated with a variety of biologic phenomena. There may be a diminishment of the cellular capacity for recovery from what otherwise would be sublethal radiation damage, with a reduction in the values of the quasi-threshold and extrapolation number. This leads to either a greater proportional cell kill than in the unsensitized state or to a change in the slope of the exponential part of the survival curve. Another cause for a potentiating effect could be a partial age synchronization of cells within the mitotic cycle so that subsequent radiation, if administered at the more sensitive part of the cycle, will be more effective.

Another approach to combining chemotherapeutic agents with radiation treatment is an adjunctive therapy wherein the drugs and radiotherapy kill tumor cells in either an additive manner or the drugs kill tumor cells in distant metastases not exposed to the radiation beam. In the attempted development of predictive model systems for the drug-irradiation interaction, this approach has been relatively neglected.

Elkind (15) has elegantly outlined some of the principles for a rational, cell-based development of combined radiotherapy-chemotherapy. He feels that three kinds of properties must be examined, ie, the responses of the cell to one or more radiation schedules, to one or more specific drugs, and to combinations of these two modalities. Since response is dependent upon the cell-cycle position of dividing cells concerned, Elkind defines this as the *age-response*

function and terms the three properties as the age-response functions of irradiation and drug action separately and in combination.

It is well established that the efficacy of cell killing by radiation is age-specific, with the age-responses having two survival maxima and minima. With chemotherapy, no such generalization can be made although cell killing by many drugs is known to be cell-cycle dependent to some degree.

Elkind (15) postulates three broad situations in which the age-response functions of radiation and drugs might interact: *(a)* overlapping age-response functions, *(b)* complementing age-response functions, and *(c)* intracellular damage interaction. In the *overlapping age-response* function, the age-dependent killing patterns of irradiation and a drug have maxima and minima at the same age. In this situation the optimal cell kill will occur when the drug and radiation are administered at intervals sufficient to allow the survivors of one agent to progress to an age at which they are sensitive to the second agent. With this overlapping interaction it is essential to have a knowledge of the changes in rates of cell aging which may result from treatments with one or both agents. *Complementing age-response* functions do not overlap, or overlap only in part, and the treatments given together can amplify each other. In common with overlapping age-response, the timing of treatments may, in a given instance, lead to greater or lesser killing effectiveness. *Intracellular damage interaction* can occur independently of overlapping or complementing age dependencies. A specific drug may impede or prevent the repair of potentially lethal or sublethal radiation damage, or vice versa, thus enhancing response.

All of this becomes even more complicated when we consider the problems of specific agents administered in multiple doses, since clinically, they almost always are. Most drugs are schedule dependent for their killing effect and it is well known that radiation fractionation permits sublethal damage repair between doses. The effects of this, over a course of therapy, require considerably larger total doses for a given effect than would otherwise be needed.

Finally, as Elkind points out, a cell based analysis of single and combination effects of one or more agents is only a starting point. The problems of tumor cell heterogeneity relative to degree of oxygenation and status of cyclic division arise both in regard to response to drugs as well as to irradiation and with regard to the ability of a drug to reach the target cells. In addition, effects on normal tissue and the applicability of supportive therapy in a given instance remain of critical importance as factors modulating the above considerations.

Screening systems for the preclinical evaluation of combinations involving chemotherapy and radiotherapy may be in vitro or in vivo or a combination thereof. The purpose of these studies may be either to uncover a potentially positive interaction between radiotherapy and drugs drawn from a selected universe of antitumor agents or to elucidate how best a selected group of single drugs and radiotherapy might interact with the goal of aiding investigators who might be attempting to clinically combine them.

The DCT currently has investigational studies of some type in progress with over 100 anticancer drugs, all of which either have proven or potential anticancer activity. It might be desirable to use screening systems to consider testing these drugs in some manner in combination with radiotherapy to uncover a potential positive interaction with the latter modality. Drugs exhibiting this potential would be considered for further, more detailed, preclinical investigation to confirm the positive interaction. Ultimately, they could be considered for clinical evaluation with irradiation in an attempt to validate the predictions of the experimental model.

There are several drugs with proven cytotoxic activity against a wide range of radioresponsive tumors. These already have been tested to some extent with radiotherapy, without positive results. Recognizing the manifold variables of schedule, sequence, and ratio, there are at least 300 ways a single drug could be combined with radiotherapy in the treatment of any individual tumor type. Given the massive number of possible therapeutic combinations, it is clear that only a tiny fraction can be actually evaluated in patients. Systems could be developed that might aid the clinician in selecting a potentially optimum sequence, schedule, and ratio of drugs and irradiation.

Conclusion

A wide range of combined modality trials are currently in progress in solid tumors (table 1). These 48 trials employ a vast array of drugs, alone and in combination, and immunotherapeutic tools in combination with surgical treatment and/or radiotherapy.

Clinical oncologists, regardless of their specific modality interests, are becoming very optimistic about the "curative" potential of combined modality therapy. They are asking many questions that the experimentalist can seek to answer while he is developing his own data, thus creating a mutually beneficial interactive approach. The DCT has initiated efforts and will continue to launch new endeavors to expand the experimental data base for combined modality studies.

TABLE 1.—Combined modality trials currently in progress

Tumor site or type	No. of overall trials	Regimens*			
		Surgical treatment plus—			
		Chemotherapy	Immunotherapy	Chemotherapy + immunotherapy	Radiotherapy + chemotherapy
Colon	4	5-FU Methyl-CCNU + 5-FU Methyl-CCNU + 5-FU + vincristine	MER BCG	Methyl-CCNU + 5-FU + vincristine + MER 5-FU + BCG	—
Pancreas	4	5-FU CCNU + 5-FU	—	—	4000 Rads + 5-FU 6000 Rads + 5-FU
Stomach	3	5-FU Methyl-CCNU + 5-FU (2)	—	—	5000 Rads + 5-FU 5-FU + methyl-CCNU
Breast	8	Melphalan (5) Melphalan + 5-FU CMF (2) CFP CMFV CMF + tamoxifen CMFVP	—	CMF + MER CMF + BCG (2) CMF + BCG + allogeneic cells	Radiotherapy + CFP
Lung (epidermoid)	4	CCNU CCNU + hydroxyurea (2)	BCG	—	Radiotherapy + CCNU + hydroxyurea
Lung (small cell)	4	CCNU + hydroxyurea CCNU + cyclophosphamide + methotrexate	BCG	—	Radiotherapy + CCNU + hydroxyurea Radiotherapy + CCNU + cyclophosphamide + methotrexate
Melanoma	11	DTIC (4) Methyl-CCNU	BCG (6) BCG + allogeneic cells (3) *C. parvum* BCG + "unblocking" plasma BCG + "normal" plasma	DTIC + BCG (2) DTIC + BCG + allogeneic cells	—
Ovary	2	Melphalan (2)	—	—	Radiotherapy + melphalan
Cervix	1	—	—	—	Radiotherapy + hydroxyurea
Brain (glioma)	3	BCNU Methyl-CCNU	—	—	Radiotherapy + BCNU (3) Radiotherapy + CCNU Radiotherapy + methyl-CCNU
Testicular	2	Actinomycin D + chlorambucil Vinblastine + bleomycin	—	—	Radiotherapy + actinomycin D + chlorambucil
Esophagus	2	—	—	—	Radiotherapy + bleomycin (2) Radiotherapy + hydroxyurea
Totals	48	25	9	7	15

*Numbers in parentheses refer to cases of two or more trials in progress with the same regimen.

LIST OF COMPOUNDS

Actinomycin D: NSC-3053

BCNU: NSC-409962; CAS reg. No. 154-93-8; urea, 1,3-bis(2-chloroethyl)-1-nitroso-

Bleomycin: NSC-125066; [2,4'-bithiazole]-4-carboxylic acid, 2'-(2-aminoethyl)-, monohydrate

CCNU: NSC-79037; CAS reg. No. 13010-47-4; urea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitroso-

CFP = cyclophosphamide, 5-FU, and prednisone

Chlorambucil: NSC-3088; CAS reg. No. 305-03-3; [*p*-[bis(2-chloroethyl)amino]phenyl]-

CMF = cyclophosphamide, methotrexate, and 5-FU

CMFV = cyclophosphamide, methotrexate, 5-FU, and vincristine

CMFVP = cyclophosphamide, methotrexate, 5-FU, vincristine, and prednisone

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate

DTIC: NSC-45388; CAS reg. No. 4342-03-4; imidazole-4-carboxamide, 5-(3,3-dimethyl-1-triazeno)-

5-Fluorouracil: NSC-19893; CAS reg. No. 51-21-8; 5-FU

Hydroxyurea: NSC-32065; CAS reg. No. 127-07-1

Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L-; L-phenylalanine mustard

MER: NSC-143769; methanol extraction residue of BCG

Methotrexate: NSC-740; CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-

Methyl-CCNU: NSC-95441; CAS reg. No. 33073-59-5; urea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitroso-

Prednisone: NSC-10023; CAS reg. No. 53-03-2; pregna-1,4-diene-3,-11,20-trione, 17,21-dihydroxy-

Tamoxifen: NSC-180973

ThioTEPA: NSC-6396; CAS reg. No. 52-24-4; phosphine sulfide, tris(1-aziridinyl)-

Vinblastine: NSC-49842; CAS reg. No. 6449-03-2; vincaleukoblastine, sulfate (1:1), monohydrate

Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1)

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Management and Analysis of Mortality Data Obtained by Treating Small Experimental Animals With Anticancer Drugs[1]

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SUMMARY

The LD10 of an anticancer agent in mice is a useful quantitative value for selecting doses to be used in therapeutic trials against experimental tumors and estimating acceptable dose levels for trials in man. A computer-based system for managing historical and current mortality and weight loss data with small animals treated with anticancer agents has been developed. The system currently has information about 1803 drugs and can produce reports of LD10s based upon all data pertaining to a given drug administered by some specified treatment regimen. The system is described, and examples of the reports that can be produced are shown.

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Since 1960, toxicity testing of normal rodents has been performed, either as parallel controls with therapeutic trials in tumor-bearing animals or as dosage tests to determine safe levels for subsequent therapeutic evaluation. Mortality data relating to 1803 drugs with assigned NSC numbers have been collected. Additionally, data for 112 chemical agents without NSC numbers and diluents (or vehicles) used for providing an injectable drug preparation have been recorded. Excluding combination toxicity tests, these data comprise 52,911 dosage tests, each test consisting of the administration by a specified route and schedule of a given dose of drug to a group of (typically) ten animals. It also represents 5645 schedule tests, each test consisting of at least one dosage test. To date, 364 unique schedules have been evaluated.

The LD10 of a drug for normal nontumor-bearing mice has been useful for planning the evaluation of that drug as a therapeutic agent against experimental tumors (1,2). For any given schedule and route the maximum tolerated dose is equal to or slightly less than the LD10 (3). The LD10s are also useful for selecting a test set of possible dose combinations for the evaluation of therapeutic effectiveness of multiple-drug chemotherapy (4-6).

Correlations between LD10s (expressed as mg/m^2) in rodents and maximum tolerated doses in man for drugs given on similar schedules have suggested the usefulness of the rodent LD10s for estimating acceptable dose levels for clinical trials in man (7,8). However, the estimates obtained for man by extrapolation from the animal data must be applied with caution. The usefulness of animal toxicity data is dependent upon its availability and accuracy. This report describes a computer-based system for the management and analysis of the animal mortality data briefly described above. Also included are sample pages of computer-produced reports which show the results of analysis and the method of presentation.

MATERIALS AND METHODS

Although the details of a particular test may vary somewhat, the general procedure for toxicity testing is as follows. Preliminary tests were performed with each drug to select a physiologically acceptable diluent (or vehicle). If possible, a vehicle forming a complete solution with the drug was used; otherwise, the drug was suspended in a "suspending vehicle" such as hydroxypropyl cellulose (Klucel). If needed, a surfactant such as Tween-80 was added to stabilize the emulsion.

The dose range to be tested was based upon previous experience (if any) with the drug, in which case

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the dose interval was usually a factor of three. For the first testing of a drug, doses were spaced by a factor of 10-100 to gain preliminary information about the approximate toxic and nontoxic dose levels. A series of drug solutions or suspensions was prepared with 0.1 ml being equal to the dose for each 10 g of body weight.

Doses of drug solutions or suspensions were then given. These doses were based on individual body weight, except for some early toxicity testing and all concurrent toxicity controls in therapeutic trials where doses were based on average body weight of the animals in the test group. Deaths were recorded daily. Animals living beyond the observation period (typically 30-60 days after the end of treatment) were scored as survivors and subsequently killed. Average body weights were determined at least weekly during the observation period.

Animals were usually housed in groups of ten and were allowed food and water ad libitum, except for oral testing with fasting, in which case food was removed overnight before administration of the drug the following morning.

The computing system utilized was a Digital Equipment Corporation PDP-15/76 Unichannel with 32K of 18-bit magnetic core, floating-point hardware, one 10.2M word disk, two 1.2M word disk units, one magnetic tape drive, card reader, line printer, and incremental plotter.

DATA BASE AND CODING

The 19 data items currently included for each experiment entered into the data base are as follows: unique experiment identification; memo number; test animal; sex (M, F, X); source of animals (supplier); date experiment started; documentation code; drug identification (NSC number); drug sample number (if available); drug lot number; diluent (if used); drug solubility in diluent; schedule; route of administration; dose(s); dose units (mg/kg/dose, etc); survivors/total; observation period; and maximum weight loss and time of occurrence. To the degree possible, internal data codes are based upon "Instruction 14, Screening Data Summary Interpretation and Outline of Current Screen" dated November 1973 (9).[7] The source of each set of data is identified by an experiment number. The memo number identifies the document describing the experimental design. The test animal (host) is identified by one of 15 codes listed in table 1. Codes for other test animals may be added as needed. The supplier and sex of the animals are also specified. "X" indicates that both male and female animals are included in the test group. The documentation code references textual information which supplies details about experimental technique or data not otherwise coded, or which calls attention to specific items of missing information.

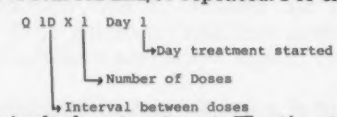
[7]Updates of Instruction 14 are available from the Drug Evaluation Branch, Drug Research and Development Program, DCT, NCI, NIH, Blair Bldg, 8300 Colesville Rd, Silver Spring, Md 20910.

TABLE 1.—Computer printout symbols and internal codes used to identify animals in toxicity tests

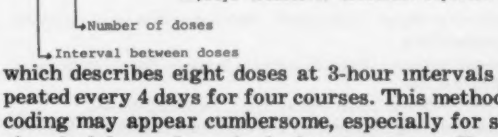
Internal code	Printed symbol or abbreviation	Host
1	SWISS	Swiss mouse
2	BDF/1	BDF_1 mouse
4	DBA/2	DBA/2 mouse
6	CDF/1	CDF_1 mouse
7	C3H/HE	C3H/He mouse
9	BALB/C	BALB/c mouse
18	AKR	AKR mouse
37	AKD2F1	$AKD2F_1$ mouse
38	D2AKF1	$D2AKF_1$ mouse
49	CD8F1	$CD8F_1$ mouse
50	RB RAT	Random-bred albino rat
51	F RAT	Fischer 344 rat
53	L RAT	Lewis rat
70	HAM	Syrian hamster
86	B6C3F1	$B6C3F_1$

Drugs are identified by NSC number. Sample and lot number (if available) may also be entered. Whether the drug forms a solution or a suspension with the diluent is indicated by the codes "1" (soluble), "4" (insoluble), or "7" (unknown).

Treatment schedules are represented by the interval between doses, number of doses, and the time treatment is started and/or repeated. For example



indicates single-dose treatment. The time treatment is started or repeated is relative time and can be expressed either as "Day" or "Hour." The interval between doses may be expressed as days (D), hours (H), or minutes (M). Another example is



which describes eight doses at 3-hour intervals repeated every 4 days for four courses. This method of coding may appear cumbersome, especially for simple schedules such as single-dose treatment. However, sufficient information is included to compute the relative time at which each dose is given. This is advantageous, for example, in identifying the time at which doses are given in a combination test or for

discovering schedules which may differ only by the relative time at which the first dose is given.

A slight modification of this format is required to accommodate treatment by constant perfusion. Perfusion for 24 hours would be coded as

Q24H × 1 Day 1

where the interval field specifies the duration of infusion. The dose associated with this schedule specifies the total dose given over the duration of perfusion. If required, the perfusion rate may then be computed.

Routes of drug administration, abbreviated symbols, and internal codes are listed in table 2. Other routes and codes may be added as required.

Dosages not expressed as mg/kg/dose are identified on printouts by the symbols listed in table 3.

The end point of the toxicity studies is expressed as a ratio of animals surviving to some specific time after the beginning of treatment to the total number of animals originally in the test group. Nonlethal toxicity is expressed as the maximum observed loss of body weight (g) and the time it occurred relative to the beginning of treatment.

TABLE 2.—Computer printout abbreviated symbols and internal codes for routes of drug administration

Internal code	Printed symbol	Route
1	IP	Intraperitoneal
2	SC	Subcutaneous
3	PO	Oral without fasting
4	CP	Constant perfusion
5	IV	Intravenous
6	IM	Intramuscular
7	OF	Oral with fasting
8	IC	Intracerebral
9	IT	Intrathoracic
10	ID	Intradermal
11	TR	Intratracheal

TABLE 3.—Computer printout internal codes and symbols used to identify dosage units

Units/dose	Symbol	Internal code
Mg/kg	Blank	1
Ml/mouse	/	5
Mg/mouse	@	4
Ml/10 g	=	6
IU/mouse	*	2
VU/mouse	#	7

SYSTEM DESIGN

In designing the structure of the computer files for accommodating the toxicity data, two requirements were considered necessary. One of these was the capability of storing and having access to data records of varying length. This capability was predicated by the need to accept the results of both single-drug and multiple-drug toxicity studies, and by the fact that the composition of any given data record would be dependent upon the schedule, number of drugs, and number of doses given. The second requirement involved the capability of adding new records as data became available and the ability to associate all records having the same specified characteristics, ie, to sort and merge by a set of keys. These requirements were fulfilled by considering the master data file as a continuous set of disk-resident contiguous records. The beginning word address of each record was placed in an associated index file. As a result, the variable-length records of the master file could be accessed either sequentially or randomly by obtaining the appropriate index.

Figure 1 summarizes the contents of a data record in the master file. Each record begins with a marker ($) and contains three data items which specify the quantity of data included. This is necessary to properly retrieve data items and to access records sequentially. For experiments in which the data cannot be included in one record, the "header information" may be referenced rather than repeated.

For each record placed in the master file, a pointer or index is preserved. The pointer is the word address in the file where the record starts and is associated with the drug or drugs used in the test. The "drug file" is a random-access file and contains the NSC number of all drugs for which toxicity data are included in the master file. The records are arranged in ascending order by NSC number and are located by a Fibonacci search.

A record of the drug file is shown in figure 2. The record may contain a chemical name or an accepted abbreviation of up to 50 characters. If there is only one index (ie, one set of data, or one record, in the master file) for the drug, the index is stored in the drug file. If there is more than one index, a pointer in the drug file refers to a record in an index file where the indices are preserved. Records of the single-drug index file are shown in figure 3. Each record may contain up to 124 indices. If there are more than 124 indices, additional records are dynamically allocated and linked as required. The "forward link" (record number of next record containing indices for the specified drug) of the last record is set to "0." The "sort switch" is incremented from a zero value for each new index added to the group. The nonzero value signals that the group of indices requires sorting.

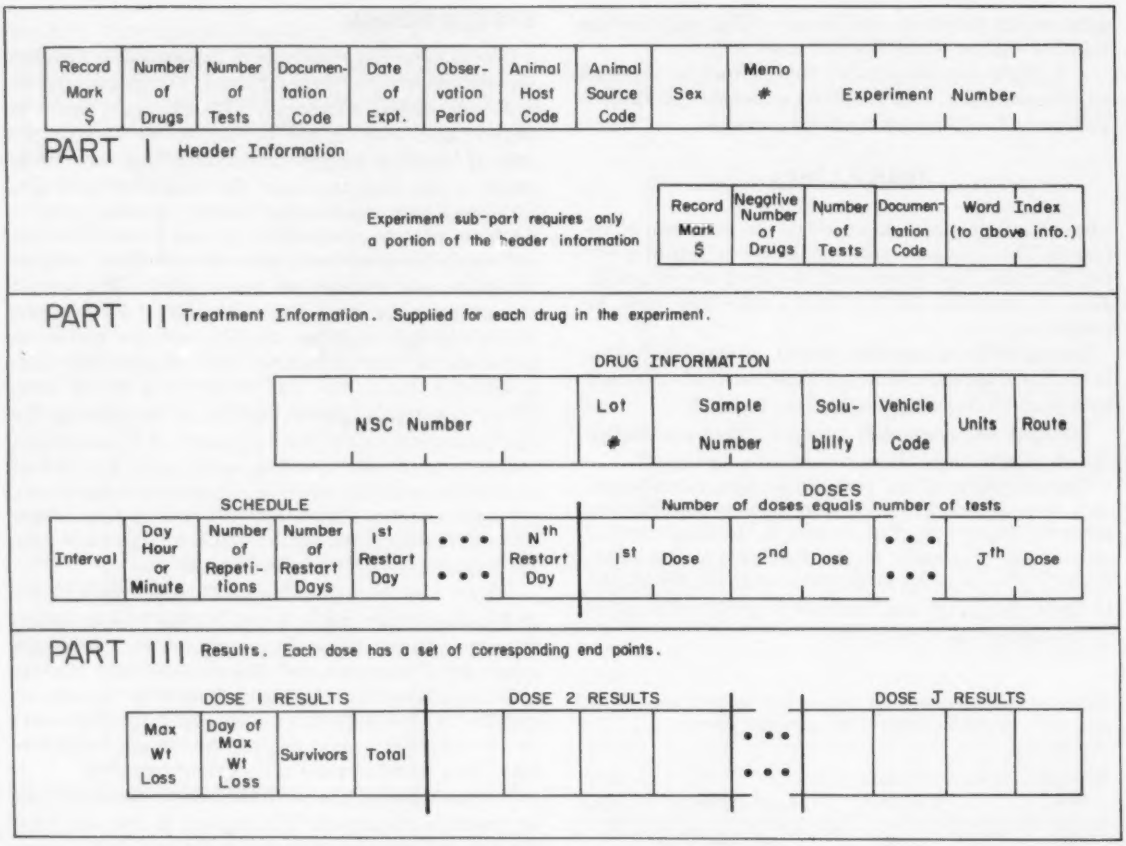


FIGURE 1.—Layout of a data record in the master toxicity file. Note that the length of the record is dependent upon the treatment schedule(s) and the number of dosage tests.

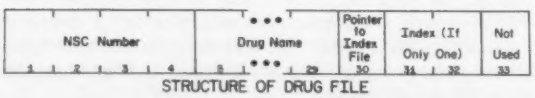


FIGURE 2.—Structure and contents of a drug file record. If there is only 1 index, the pointer is set to "-1" and that index follows. If there are no data for this drug, then the pointer is set to "0."

Indices for combination toxicity data are placed in another file, a record of which is shown in figure 4. This file must be searched to locate indices for a particular drug combination, which is identified by a set of NSC numbers.

Figure 5 outlines the general logical flow and procedures required to process the results of toxicity studies. The data items described earlier are first transcribed to preformatted coding forms from which punched cards are prepared. Each set of cards is edited for logical and format errors and corrected as necessary. The "error-free" data sets are passed to an update program which processes the cards and

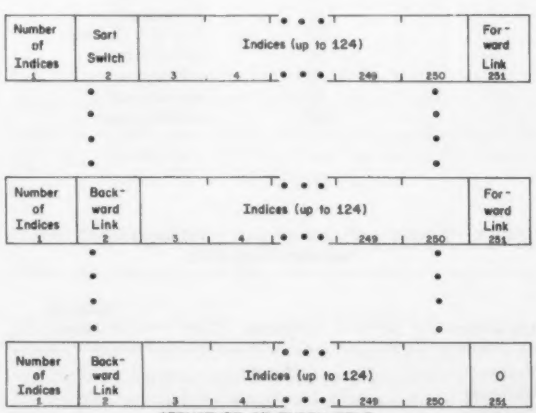


FIGURE 3.—Structure and contents of a linked set of records in the index file for single-drug toxicity data. If there is only 1 record in the group, then the "forward link" is set to "0."

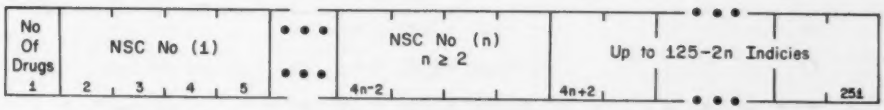


FIGURE 4.—Structure and contents of an index file record for the combination toxicity data.

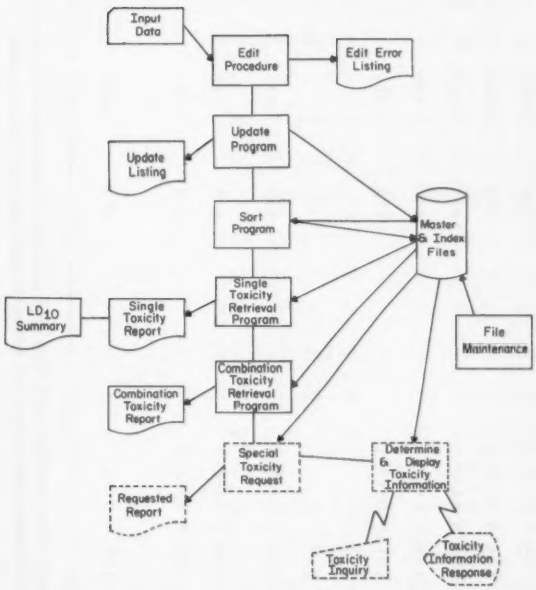


FIGURE 5.—System flowchart for management, analysis, and reporting of toxicity data obtained with small experimental animals. The broken lines indicate possible extensions to the existing processes, such as special requests or an on-line inquiry and reporting procedure.

places the output data onto the master file. The update program also appends the data pointer to the proper record of the single-drug or combination toxicity index file. Each set of indices is then sorted by some set of characteristics, typically specified by treatment schedule, route, and test animal. Additional characteristics could be specified, such as sex or drug diluent. The sorted set of indices then provides the mechanism for accessing and merging data of similar characteristics and preparing suitable reports.

All main programs were written in FORTRAN for the PDP-15/76 and, as a consequence, embody some machine-dependent code. Several utility subroutines were coded in assembler language in order to decrease execution time and increase flexibility.

DATA ANALYSIS AND REPORTING

Toxicity data of similar characteristics are identified and located through a group of indices to the master file. The entire set of doses and corresponding surviving fractions (F_s) are examined as indicated in table 4. If the set of data meets the conditions indicated, then a probit analysis (10) is performed on $\log_{10}$ (dose) and F_s. The results of the probit analysis, LD10, LD50, LD90, or any other lethality desired, are then further examined as described in table 4. Confidence limits at the 95% level may also be computed for each lethality. A report of LD10s, LD50s, and LD90s is prepared which incorporates a list of all experiments from which the values are computed. A sample page of this report is reproduced in figure 6. If the lethalities cannot be computed, appropriate messages are printed. This report

TABLE 4.—Conditions detected and action taken after editing toxicity data

Condition*	Action
$F_s = 0$ for all doses	LD10 < lowest dose†
$F_s = 1$ for all doses	LD10 > highest dose†
Only 1 dose tested (even if replicated) and $0 < F_s < 1$	Insufficient testing†
$n < 3$ and $0 < F_s < 1$	Insufficient testing†
n > limit	Combine all tests with identical doses and F_s
n > limit (after preceding merge)	Combine all tests with identical doses
$3 \leq n \leq$ limit	LD10, LD50, LD90 found by probit analysis‡
Examine probit analysis results	
1. Negative slope	1. Unsatisfactory data
2. LD10, LD50, or LD90 < lowest dose tested	2. Indicate lethality < lowest dose tested
3. LD10, LD50, or LD90 > highest dose tested	3. Indicate lethality > highest dose tested

*F_s = fraction of animals surviving test; n = total number of dosage tests.

†Probit analysis is not performed.

‡Probit analysis performed with F_s and $\log_{10}$ (dose).

EXPT NO	DC	DATE	HOST	SEX	VEH	SAMP	RTE	SCHEDULE	MG/KG/DOSE LD10	LD50	LD90	TOTAL TESTS
ST2H5		1- 6-65	SWISS	M	8		IP	Q 1DX 1 DAYS 1	132	315	753	6
ST6		9- 9-60	SWISS	F	7		IP	Q 1DX11 DAYS 1	<250	273	388	3
707		11- 6-68	BDF/1	M	25	MA00	IP	Q 3HX 8 DAYS 1	73	>75	>75	4
707		11- 6-68	BDF/1	M	25	MA00	IP	Q 3HX 8 DAYS 1,9	73	>75	>75	4
707		11- 6-68	BDF/1	M	25	MA00	IP	Q 3HX 8 DAYS 1,5,9	73	>75	>75	4
LE585		2-11-65	BDF/1	M	8		IP	Q 1DX 1 DAYS 1	357	443	550	11
LE694		5-27-65	BDF/1	F	8							
LE515		11-11-64	BDF/1	F	8							
707		11- 6-68	BDF/1	M	25	MA00	SC	Q 1DX 1 DAYS 1	514	>600	>600	4
707		11- 6-68	BDF/1	M	25	MA00	PO	Q 1DX 1 DAYS 1	720	>800	>800	4
707		11- 6-68	BDF/1	M	25	MA00	IV	Q 1DX 1 DAYS 1	250	278	310	4
707		11- 6-68	BDF/1	M	25	MA00	IP	Q 1DX 5 DAYS 1	330	>400	>400	4
707		11- 6-68	BDF/1	M	25	MA00	IV	Q 1DX 6 DAYS 1	>200	>200	>200	4
707		11- 6-68	BDF/1	M	25	MA00	IP	Q 1DX 9 DAYS 1	330	>400	>400	4
707		11- 6-68	BDF/1	M	25	MA00	SC	Q 1DX 9 DAYS 1	343	>400	>400	4
707		11- 6-68	BDF/1	M	25	MA00	PO	Q 1DX 9 DAYS 1	>400	>400	>400	4
LL8		12-27-67	BDF/1	X	26		IP	Q 1DX11 DAYS 1	>50	>50	>50	1
CT631		7-31-68	BDF/1	F	26		IP	Q 1DX15 DAYS 1	215	321	479	27
455		7- 1-66	BDF/1	F	8							
CT608		3-11-68	BDF/1	F	26							
CT569		3-14-67	BDF/1	F	26							
461		7-20-66	BDF/1	F	26							
633		11- 9-67	BDF/1	M	8	MA00	IP	Q 4DX 3 DAYS 1	439	487	539	9
707		11- 6-68	BDF/1	M	25	MA00						
707		11- 6-68	BDF/1	M	25	MA00	IP	Q 8DX 2 DAYS 1	375	418	465	4
RT243	3	11- 8-63	RB RAT	M	8		IP	Q 1DX 7 DAYS 1	176	271	419	8
RT202	3	6-11-63	RB RAT	M	8							
RT254	3	12- 4-63	RB RAT	M	8							
HT152	3	5-11-63	HAM	F	8		IP	Q 1DX 7 DAYS 1	279	576	>1000	4

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FIGURE 6.—Sample page of the computer printout of the single-drug toxicity report. This page gives the computed lethalities for 6-chloropurine (NSC-744). > = Lethality is larger than the highest dose tested; < = lethality is below the smallest dose tested.

NSC NO	HOST	SEX	DOSE TESTS	RT	SCHEDULE	LD10 MG/KG/DOSE
750	BDF/1	X	35	IP	Q 1DX 7 DAYS 1	79
750	BDF/1	X	25	IP	Q 1DX11 DAYS 1	55
750	BDF/1	M	5	IP	Q 2DX10 DAYS 1	35
750	C3H/HE	X	11	IP	Q 1DX 1 DAYS 1	40
750	C3H/HE	F	3	SC	Q 1DX 5 DAYS 1	35
750	C3H/HE	X	11	IP	Q 1DX11 DAYS 1	7.0
750	RB RAT	M	4	IP	Q 3HX 8 DAYS 1	<8.0
750	RH RAT	M	4	IP	Q 3HX 8 DAYS 1,9	<8.0
750	RB RAT	M	4	IP	Q 3HX 8 DAYS 1,5,9	<8.0
750	RB RAT	M	4	IP	Q 1DX 1 DAYS 1	<64
750	RB RAT	M	4	OF	Q 1DX 1 DAYS 1	<64
750	RB RAT	X	8	IP	Q 1DX 7 DAYS 1	15
750	RB RAT	M	4	IP	Q 1DX 9 DAYS 1	<8.0
750	RB RAT	M	4	OF	Q 1DX 9 DAYS 1	<8.0
750	RB RAT	M	4	IP	Q 4DX 3 DAYS 1	<32
750	RH RAT	M	4	IP	Q 8DX 2 DAYS 1	<64
750	HAM	X	24	IP	Q 1DX 7 DAYS 1	60
751	SWISS	X	54	IP	Q 1DX 7 DAYS 1	66
751	SWISS	M	5	IP	Q 2DX 7 DAYS 1	>210
751	BDF/1	X	33	IP	Q 1DX 7 DAYS 1	16
751	BDF/1	X	33	IP	Q 1DX11 DAYS 1	41
751	HAM	M	14	IP	Q 1DX 7 DAYS 1	744
752	SWISS	X	39	IP	Q 1DX 1 DAYS 1	28
752	SWISS	X	10	IP	Q 1DX 7 DAYS 1	2.8
752	SWISS	X	11	IP	Q 1DX11 DAYS 1	0.9
752	BDF/1	M	2	IP	Q 1HX 8 DAYS 1	>0.6

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MYLERAN : 750
DL-ETHIONINE : 751
6-THIOGUANINE : 752

FIGURE 7.—Sample page of the LD10 summary report. The text describes the arrangement of the report produced as a computer printout. Tables 1, 2, and 3 serve as keys for interpreting "host" (test animal), "RT" (route), and any symbols printed to the right of the LD10. A "dose test" is a group of test animals which receive a specified dose of drug given by some route and schedule.

serves as a basis for verifying the completeness of the data base and examining for errors and omissions.

A report listing only LD10s is also prepared (fig 7). The report is arranged in NSC number order, and for each NSC number the results are grouped according to test animal. Further, within each test animal group, treatment schedules are arranged in an orderly manner by interval between doses, number of repetitions, and number of restarts, and are subdivided by route of administration. If drug names or abbreviations are available, they are listed at the bottom of each page. The entire report is in excess of 200 pages and has been reproduced and bound together with an alphabetic listing of drug names.[8]

Although combination toxicity data are available on the disk files, they cannot be analyzed or reported in the same way as has been done for the single-drug toxicity data. Before any analysis of the combination toxicity data can be performed, an extension to the present system will require implementation. When this is accomplished, it may be suitable to perform an analysis as suggested by Skipper (4). Let

$$f_{ij} = D_{ij}/LD_{10}^{i}, \quad i = 1,\ldots,n$$

where D_{ij} is the jth dose of drug i in an n-drug combination and LD_{10}^{i} is the LD10 for drug i when used alone on the same schedule. Thus, f_{ij} represents the fraction of the single-drug LD10 employed in the combination. Let

$$F_j = \sum_{i}^{n} f_{ij}$$

be the sum of the fractions of individual LD10s used in the combination. A probit analysis may now be performed with the F_j and the corresponding mortality data to produce F10, F50, and F90, which represent the values of the sum of fractions of LD10s that produce 10%, 50%, and 90% mortality. If F10 is > 1 then the toxicity of the combination is probably less than additive. Thus, the value of F10 could serve as a flag to suggest drug combinations which have less than additive toxicity.

Other reports are periodically generated which provide statistics about the source, quantity, and extent of the data, listings of drug names available, lists of indices, list of unique schedules, etc. These reports also serve as a means for error detection.

The maximum weight loss and the time of occurrence do not appear on any of the reports described, but these could be modified or new reports could be generated which include indications of nonlethal toxicity.

DISCUSSION

The computerized system for the management and analysis of toxicity data centers around disk-based, random-access, variable-length data records. Access to these records may be sequential or random via indices preserved and partitioned by NSC number. Maintaining and accessing the variable-length records required more complicated programming than would have been necessary for fixed-length records. However, the variable-length records provided considerable flexibility for the content of each record and utilized disk storage quite efficiently. Output to the file was achieved with the aid of a subroutine which took care of the necessary "bookkeeping."

The indices provided the means of tag-sorting the file. The indices within each drug were grouped according to characteristics of the data to which they referred. Reports were then prepared by examining and processing the data referred to by each group of indices.

Three modifications to the system are planned. One is the implementation of schedule codes. Each code will represent a record in a file which describes the schedule. This will reduce storage requirements in the master file and accommodate schedules not able to be expressed in the present format. The proposed procedures for analyzing the combination toxicity data will require the addition of a file containing all of the computed LD10s. This information will be necessary to compute the fraction of LD10 used in the combination. Also, the capability of performing a probit analysis on the data of a specified experiment will be provided. A log-dose versus probit plot and printout will comprise the output.

The proposed method for processing the combination toxicity data implicitly assumes that the F_j are far more important in determining the toxic response than are the individual f_{ij} that make up the F_j, ie, the toxic response is due primarily to the sum of the fractions of the individual LD10s rather than to the relative proportion of each drug used in the combination. Thus, an infinite number of dose combinations could produce the same F_j and presumably the same toxic response. It should be feasible to check the validity of this assumption using the combination toxicity data available. Also, it is presumed that when F10 is >1 the toxicity of the combination is less than additive. This relationship may serve as a useful qualitative flag, but an exact quantitative interpretation must be considered in reference to the

[8]The complete LD10 summary will be published elsewhere. Copies of the current version may be requested from the authors.

dependence of lethality upon dose. For example, it does not necessarily follow that ½ LD10 is equivalent to an LD5.

The processing of the data includes efforts at ensuring accurate data entry. The punched cards are listed and compared to original data sheets. Any corrections are made before computer editing, which may discover additional errors. After entry, duplicate experiment numbers are investigated, unique schedules are examined for plausibility, certain statistics are generated, and data which produce suspicious results of the probit analysis are examined for causes. These efforts are probably effective in eliminating most errors.

Some caution must be exercised when using LD10s computed from historical data. It must be remembered that they result from combining the data of all experiments having similar specified characteristics. Therefore, certain factors which might affect the historical results are not taken into account, eg, drug purity may not be consistent, test animal characteristics may alter with time, laboratory techniques may change, accuracy of drug preparation may vary, etc. Also, the results presented pertain to the specified sort keys. Consequently, the LD10s computed are based upon varying amounts of data obtained at different times and will exhibit some inconsistencies. Such inconsistencies are quite often readily identified by examining the LD10s for a group of similar treatment schedules.

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Impact Upon Screeners of the Front-End Redesign[1]

William T. Miller, Jr.[2,3]

SUMMARY

This article discusses a redesign and implementation of portions of the Biological Data Processing System (which processes the Biological Screening Data) and the impact of the changes on the screeners who supply the input to the system. The role of the Data Processing Contractor to the Drug Evaluation Branch, Drug Research and Development Program, Division of Cancer Treatment, is discussed; this group is responsible for the changes. A brief explanation of the current system and its shortcomings is provided along with a statement of the objectives of the redesign. It is concluded that there will be minimal impact upon the screeners by the introduction of new data collection forms and instructions, or (in some instances) the introduction of totally new data input methods involving terminals. These changes are seen as beneficial because they will eliminate some of the data manipulation requirements necessary to compensate for the inadequacies of the existing forms.

[Cancer Chemother Rep Part 2, vol 5:253-263, 1975]

The purpose of this article is to acquaint screeners with the modifications now being implemented in the NCI's Biological Data Processing System and the impact of these modifications upon current procedures. The changes are being accomplished by the Data Processing Contractor (DPC) to the Drug Evaluation Branch (DEB), Drug Research and Development Program (DR&DP), DCT, under the direction of the Automated Information Section (AIS). Because these changes are limited to the input processing area and are not concerned with the file update or the generation of output reports, the term "front-end redesign" has been applied to the total effort.

The Role of the DPC

The primary function of the DPC is to serve as the data processing interface between the DEB and its screening laboratories. In this role, the DPC provides two important services. First, it operationally processes the test results of the individual screeners (see fig 1), records the data on the centralized master files, and distributes reports to the NCI and its screeners and suppliers. Secondly, it is responsible for the maintenance of the operational programming system and the implementation of changes to that system as requested by the Screening Section of DEB. In performing both of these services, the DPC utilizes the facilities of the Division of Computer Research and Technology (DCRT) located at NIH in Bethesda, Maryland.

In performing the first service, the DPC accepts test data from the screening laboratory in one of two forms. The data are keyed onto computer cards or tape from the appropriate data collection forms (ie, NIH 1157, 1158, etc) by the screener, or they are sent on the forms from which they must be keypunched by the DPC. In either event, the forms are manually checked for completeness to ensure that sufficient data have been captured and properly recorded prior to their entry into the computer system. Valid data result in the updating of the Biological Data Processing System master files and the generation of output reports (Screening Experiment Analysis [SEA] forms and Screening Data Summary [SDS] forms) for the NCI, the drug suppliers, and the screeners. Invalid data which are not correctable by the DPC are transmitted to the NCI for review before being returned to the submitting screener for correction and resubmission. This is a cyclic process which is currently accomplished on a biweekly basis. Every 2 weeks the DPC handles approximately 29,000 input transactions and distributes in excess

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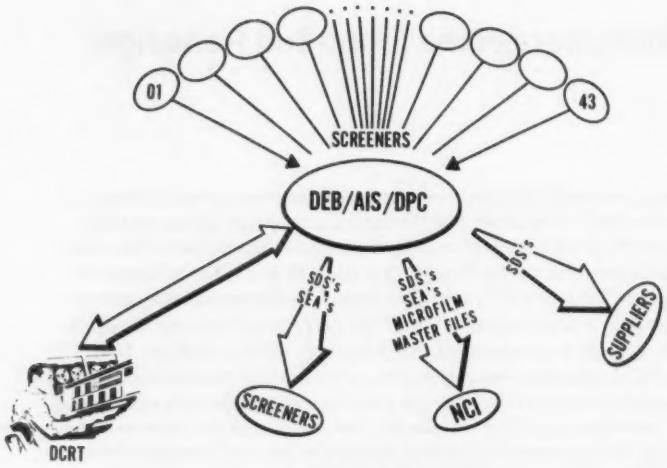

FIGURE 1.—DPC data processing interface.

of 60,000 pages of output reports to the NCI, the screeners, and some 2700 drug suppliers.

In its capacity as operations and maintenance coordinator of the Biological Data Processing System, the DPC employs systems analysts and programmers to maintain and redesign, as necessary, a system of 103 computer programs. These programs perform the required data edits and mathematical calculations, update the appropriate master files, and automatically produce the desired reports in sufficient quantity to satisfy the distribution requirement. The programs represent more than 110,000 individual instructions to the computer system prepared in five different programming languages. In addition to the computer programs, a set of master files is maintained in the form of magnetic tape with separate files being provided for natural products and synthetic test data. The synthetic test data are further subset between regular synthetic compounds and "selected agent" compounds so that those synthetics of specific interest are more accessible. The file sizes are shown in figure 2 and require 12 reels of magnetic tape to accommodate them. Figure 3 provides some indication of the volume of this data by illustrating the height of three separate columns which would result from recording the data on cards and stacking the cards. Equally impressive is the fact that 5½ miles of magnetic tape are required to store the testing data.

Of equal importance to the maintenance of the computer programs and central files is the role of the DPC with respect to the maintenance of over 4000 pages of documentation. This documentation describes the existing computer programs and their run instructions, operating procedures, and screener data recording instructions.

Central File Utilization

The primary purpose of the data processing system is to build and maintain a master file of drug testing data accessible to the NCI and those groups it may designate. The current file has been maintained continuously since 1956 and contains a history of virtually every drug ever tested within the DEB, DR&DP. The file has four basic uses (*see* fig 4) which are as follows:

1. The file permits the recurring dissemination of current test results together with a summary of the drug's previous testing history each time the drug is actively tested. This information is prepared automatically (whenever the results of new testing are submitted) and is distributed to the NCI, the screener(s), and the appropriate drug supplier(s).
2. The file permits the inquiry of historical data on either active or totally inactive compounds. Requests for information are directed to the DEB where they are converted into data inquiries yielding the desired reports.
3. The file is used as a data source for special studies, ie, when a survey of a broad spectrum of data is indicated or when it is necessary to compare data originating at various times. Most recently, the file was used to evaluate the new synthetic protocol involving the mini-screen. Because of

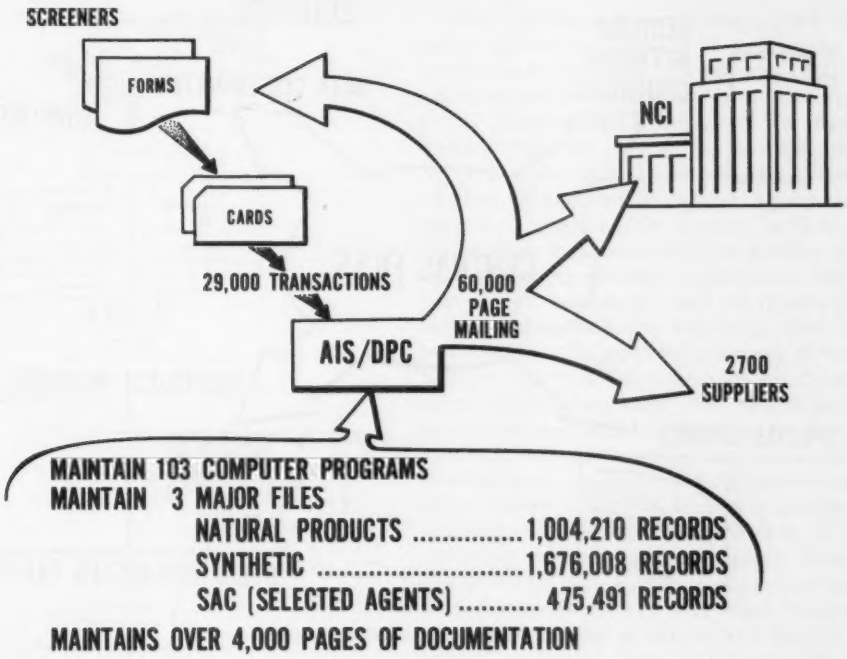

FIGURE 2.—DPC interface services.

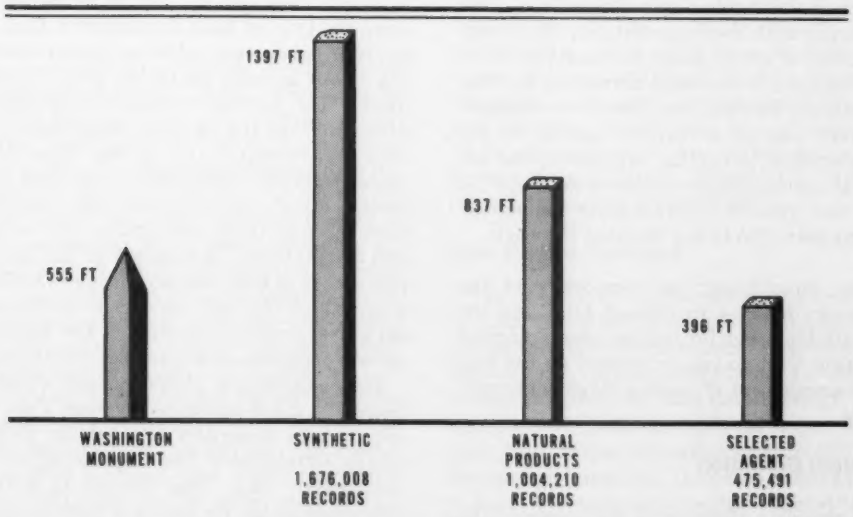

FIGURE 3.—File size representation (punch card equivalent).

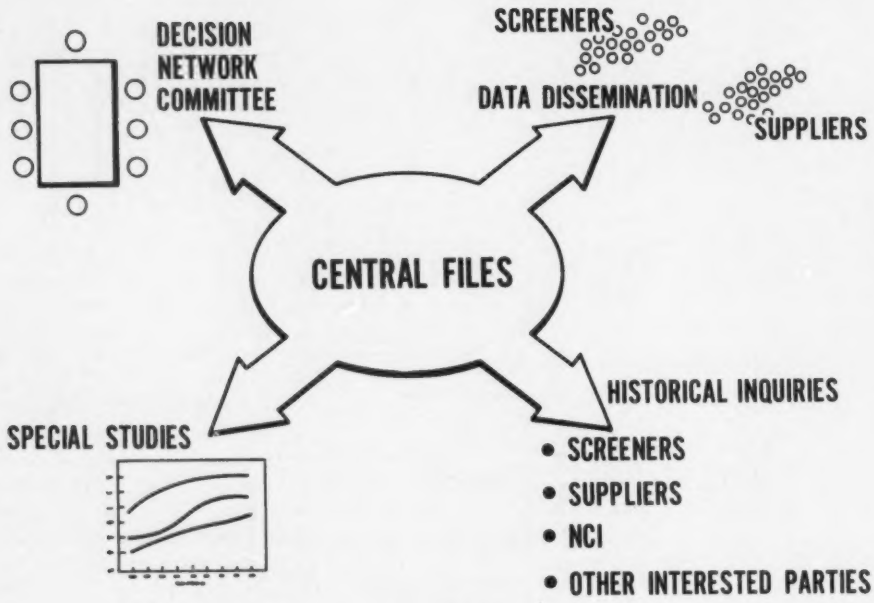

FIGURE 4.—Central file utilization.

the historical nature of the file, it is possible to develop statistics such as the program yield (number of active compounds in relation to the total number of compounds tested) involving either comparable or different test systems or protocols.

4. Finally, the file is used as an information source for the Decision Network Committee of DCT. This group, charged with the responsibility of managing potentially effective drugs through the NCI's Decision Network from initial screening to clinical trial, utilizes the data from the file to evaluate a compound's success or failure against established test profiles. A typical example of the use of the file as an evaluation criterion would be the review of test results in evaluating compounds for entering point IIA in the Decision Network.

In summary, considering the complexity of the testing hierarchy leading to clinical trial and the volume of available drugs for testing, the Biological Data Processing System can be viewed as the tool that makes management of such a large-scale program possible.

Current System Definition

In order to permit a meaningful discussion of the modifications to the current data processing system and to evaluate the impact of these modifications, it is necessary to understand the current program system design. The following is a brief discussion of the system and its shortcomings. Figure 5 provides a simplified flowchart of the current Biological Data Processing System.

All input is card-image oriented and enters the system at any one of four entry points, depending upon the type of data encountered. Basically, there are four subsystems which are essentially independent of one another up to the point of updating the master files: (*a*) the combination chemotherapy preprocessor; (*b*) the in vitro preprocessor; (*c*) the in vivo preprocessor; and (*d*) the "final edit" preprocessor (certain "other data" only pass through this preprocessor). Each preprocessor essentially processes its own data, performing the necessary edits and calculations. The output of the first three preprocessors is then passed through the fourth where a repeat of the edit process is performed and the data are properly formatted for handling by the update programs and placement on the master files.

The combination chemotherapy subsystem is, of necessity, a self-contained system. That is, it has its own update programs and master files which are totally unrelated to the single-drug in vitro and in vivo master files. This division is dictated by the differences in the formats and types of data recorded for the two systems. Until recently, a tie was more

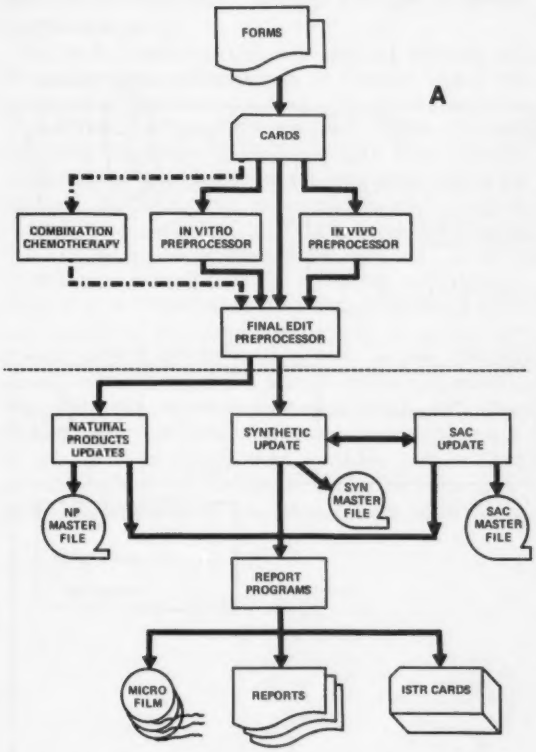

FIGURE 5.—Current Biological Data Processing System definition. A = Front-end portions of system to be replaced or modified by redesign.

apparent because single-drug testing, accomplished within a "combination control pack," was actually recorded in both systems. This has changed within the past several months so that now only a notification message is passed to the single-drug system for each NSC number encountered in a combination chemotherapy control pack. The message results in a line on the screening data summary advising, "SEE COMBINATION CHEMOTHERAPY." Thus, the SDS user is alerted that the compound has been tested in combination with at least one other compound and is directed to the combination chemotherapy report system to review the test results.

Once acceptability is determined, the data pass to the update routines in an acceptable format and are placed on the appropriate master file. The report programs are then invoked, and reports are prepared for those NSC numbers for which current testing has taken place or for which specific inquiries have been made.

While the current system has served (and continues to serve) its function of processing screening results, it is becoming less and less efficient and responsive to the changing needs of the screening program. It is inefficient because of the manual intervention required due to its multiple data entry points and the virtually independent operation of its various subsystems. It is highly form and format dependent, significantly limiting both a change in the existing forms and the introduction of new ones for new testing concepts. Additionally, the introduction of new techniques, such as remote data entry via a terminal, would be extremely difficult to implement within the current program structure. The problem of form dependence is continually being aggravated as more and more of the data fields on the current forms are rendered inadequate, either by virtue of values exceeding field limitations or a desire for more sophisticated representations of the data. Figure 6 depicts typical examples of these problems that complicate the job of recording screening data at the laboratories. When the field size is inadequate special alphabetical representation of numbers must be used which requires manual conversion by the screeners and special programming to recognize, store, and display the alphabetical representations. The best example of increased sophistication with respect to existing data fields is the interval or regimen field, where the current size and structure are no longer adequate to express the precision required for newer treatment schedules.

Figure 5(A) identifies those portions of the existing system that are being replaced or significantly modified by the redesign. Basically, the changes affect the system up to the point of updating the master files but the file design and the existing outputs (SEAs, SDSs, interim screening test record cards, etc) are unaffected. Because the current redesign attacks the input processing portion of the system, the term "front end" was introduced. All four major preprocessors are either being totally replaced or significantly altered by a totally new concept of data entry and processing.

New Design Concept

The objectives of the redesign are numerous and include the elimination of (*a*) the multiple entry points for data, (*b*) the manual intervention required between the various subsystems, (*c*) the repetitious points at which the same data undergo the same edits, (*d*) the dependence upon format or form of input data, and (*e*) the utilization of different routines for the same calculation. They also include the introduction of (*a*) a modular program concept that will enhance future modifications, (*b*) flexibility with respect to both form and format of the input data, (*c*) single-

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SCREENING TEST RECORD—IN VIVO

HOST	TUMOR	SCREEN-ER	CONTROL NUMBER	PARAM	INOC SITE	VEH.	RT	INTERVAL	PRE.	NSC NUMBER CORE	NSC NUMBER SAMPLE NO.	DAY 1ST INJ.	NO. OF INJECTIONS
1 2	3 4	5	P 0 0 1 7 8 9 10	11	12	13	14	+ 1 15 16	17	18 19 20 21 22 23	24 25 26 27	B 28	29 30

DOSE NO.	DOSE/INJ.	NUMBER OF CURES	NUMBER OF NO. TAKES	STATUS CODE TEST	STATUS CODE SUF.	SPEC. STUDY CODE	NO. OF ANIMALS	DATE ON YEAR	DATE ON MONTH	DATE ON DAY
C 31	32 33 34 35 36 37 (−) (A)	38	39	52 53	54	55	56 57			

SURVIVORS

DAY	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
NO.																								C7
DAY	24	25	26	27	28	29	30																	
NO.																								

NO.	ANIMAL WEIGHTS INITIAL DAY (DAY_____)	ANIMAL WEIGHTS TOXICITY EVAL. (DAY_____)	TUMOR WEIGHTS
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			
15			
16			
TOTAL	40 41 42 43	44 45 46 47	5 3 2 4 1 48 49 50 51
AV.			
BODY WT. CHANGE (TOX-INITIAL DAY) + −		EVALUATION	

COMMENTS:

PHYSICAL STATE
_____SUSPENSION
_____SOLUTION

LD ( )	ED ( )	T.I.

TEST NO.	NEW TEST	TOXIC AT	(T/C)1	(T/C)2	PREVIOUS STAGE INDEX	T/C THIS TEST	NEW STAGE INDEX	CONTRACTORS NO.

NIH 1158-1 (REV. 1-73)

FINAL DAY SERVICE BUREAU COPY

FIGURE 6.—Current form problems.

source editing and calculating of data results, (*d*) single-point data entry, and (e) a single programming language.

Figure 7 illustrates this new concept whereby all data, regardless of the form or format, enter the system at a single point and are processed completely under the control of the program. While it is necessary to continue to accommodate the punched cards which result from the existing data collection methods, flexibility has been introduced to permit virtually any method of data entry supported by the NCI's data processing facility (DCRT).

Data are entered through a master control program that is responsible for recognizing each form and format of input, sorting it into its proper sequence, and directing it to one of several format processing modules. Each format processing module interprets the incoming data and "manages" their processing by reformatting as necessary and scheduling a series of independent routines such as edits or calculations which sequentially perform their unique function upon the data. These routines in turn signal their success or failure to the controlling format processing module along with appropriate error messages for an exception report when the data must be rejected. An evaluation of these signals is performed by the processing module, and a decision is made as to the acceptability of the input data. Upon establishing their acceptability, the data are formatted into records for processing by the existing update programs. Under the new scheme, only the format processing module is oriented to the format of the data. This means that a separate format processing module will be required for each new format introduced, but all of the other modular routines will be unaffected by such a new entry.

New Features

In addition to the flexibility and efficiencies to be achieved in operation and program maintenance, several new features are being introduced (see fig 8).

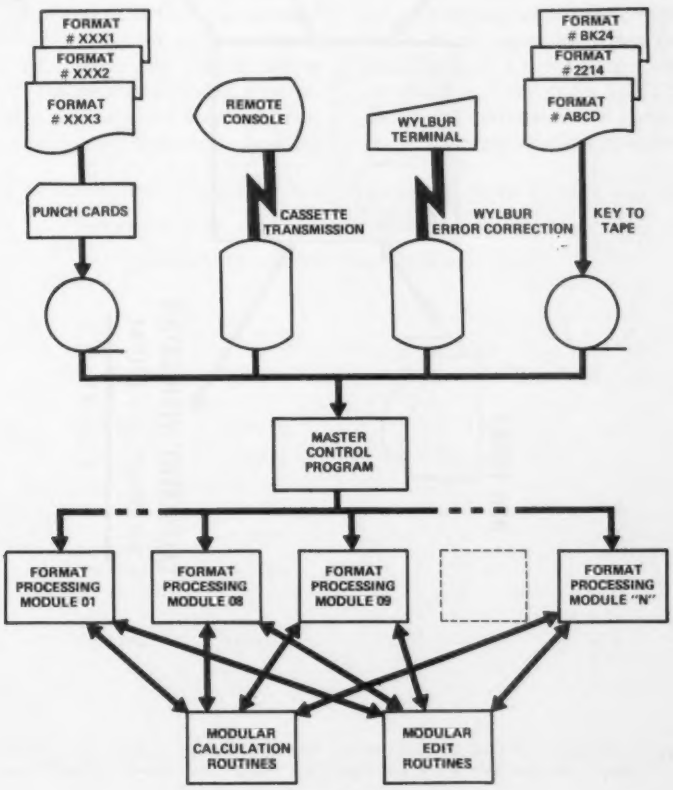

FIGURE 7.—New front-end design concept.

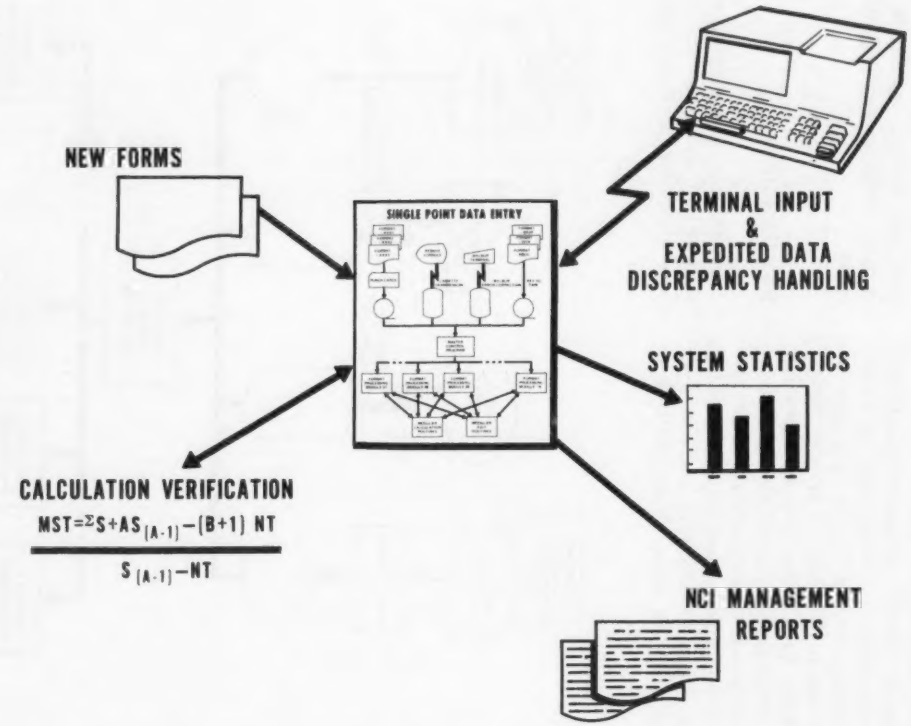

FIGURE 8.—New features.

Changes are being made so that screening laboratories with a high volume of input data can enter data into the computer through the use of data entry terminals located at their establishments. In this way, the screener can bypass the use of data collection forms and enter the data into the terminal directly from the laboratory notebooks. The terminal would supply the equivalent of the "form" by requesting information by name and then providing a specific location where it is to be entered. As an example, the terminal might request "HOST CODE" and then provide an area enclosed in brackets [--] where the two-position host code would be entered. When a complete control pack or packs are assembled on a cassette tape, a special command would transmit the data over telephone lines to a special receiver at the computer center. The data would be processed as with any other data with one significant difference, control packs rejected because of data discrepancies could be returned within a matter of hours directly to the screener's terminal. At present, the resolution of data discrepancies is a cumbersome process involving reviews by data analysts at the DPC facility, reviews by NCI personnel, and finally, return of the offending pack to the screeners' facility by mail when local resolution of the problem cannot be accomplished. Under the scheme being considered for use with the remote terminal system, the entire pack containing the data discrepancies would be returned via telephone lines to a cassette tape at the screener's terminal site. The operator would then play back the control pack on the terminal display in the same manner it was originally created. Along with the data, however, error messages would be provided directing the correction of the offending data fields. Once the errors have been corrected, the pack would again be transmitted to the computer.

The new design concept will include a facility for recurring output of management reports on system statistics. Such statistics will permit the monitoring of system barometers such as amounts and types of testing and yields compared to prior time frames or conditions. These will be automatic in that they will be output as the result of every processing run. Figure 9 illustrates such a report comparing the total tests from the current year to a similar period in the previous year. Additionally, the report indicates the yield (number of active compounds compared to total compounds tested) for the two periods and the percentage of change.

Similar to the system statistics, but more flexible, will be a provision for other NCI management reports that will permit members of the staff to monitor specific aspects of the program on an as-required basis. As an example, it may be desirable to monitor the results of a new test system, treatment schedule, or whole protocol for a short period after its introduction. The monitor will have the option of requesting certain parameters (number of tests, yield, mean

SUMMARY OF TOTAL TESTS BY MAJOR TEST SYSTEMS FOR YEAR TO DATE AND PREVIOUS YEAR TO DATE WITH CURRENT PERCENTAGE ACTIVITY COMPARED TO AVERAGE FOR PREVIOUS YEAR

DATE 06/10/75

TEST SYSTEM	- TOTAL TESTS - YEAR TO DATE	PREV YTD	- - ACTIVITY - - YEAR TO DATE	PREV YTD	PERCENT CHANGE
3LE21	33156	30120	3 %	3 %	0
3PS31	27118	22750	8 %	9 %	- 12 %
38131	500	350	5 %	4 %	+ 25 %
5WA16	7300	8010	2 %	3 %	- 33 %
OTHER	101200	98198	7 %	5 %	+ 40 %
TOTALS	169274	159428	6 %	5 %	+ 20 %

NOTE - THIS IS A SAMPLE DEMAND REPORT USING THE YEARLY STATISTICAL FILE
THE FIGURES SHOWN ARE ONLY INTENDED TO SHOW THE REPORT FORMAT

FIGURE 9.—Sample system statistics.

survival times of the control groups, etc) for an identified entity for any particular time period desired. It is envisioned that these reports will be for short durations (although no such restriction exists) and for specific situations. It is also contemplated that this facility will be used to flag "exception" conditions that, while not in error, are of concern such as: (*a*) packs where more than 25% of the tests indicate underweight animals; (*b*) packs containing inoculum levels other than five; and (*c*) control death patterns of significant variation from the norm.

Also, the new design will incorporate logic to permit the comparison of screener calculations to computer calculations to assist in detecting discrepancies in other portions of the data such as death pattern or animal and tumor weights. Whereas screener calculations have never been used in the past, they will now be entered and compared to the computer calculations. When they differ by more than a preset amount (to allow for rounding), the pack will be rejected and reviewed for input differences in either the death pattern, number of animals, or weight fields.

Finally, the new design will permit the introduction of new forms for the collection of screener data. These new forms will eliminate the numerous problems already discussed and reduce the amount of duplication required to enter identifying data on subsequent data sheets.

Implementation Schedule

Most of the changes we have discussed are already under way. It is difficult, however, to identify the exact time that the screener will be affected because of the approach being taken for verifying the new system's performance. While all of the new features and design concepts are highly desirable, even more desirable is the assurance that the new system is capable of properly handling the screening data on an operational basis. Testing of the new system is extensive, but no test can ever adequately duplicate the processing of operational data over an extended period of time. Therefore, the new system is scheduled to enter a period of parallel operation with the current system beginning in September of 1975. Operational status of the new design will occur when it demonstrates that it can handle all the data currently being processed by the existing system. As the name implies, parallel testing involves operating both systems using the same input data (*see* fig 10). Both systems receive and process the same data in the same manner but only the output of the current system is used to interface with the rest of the Biological Data Processing System. The interim results (the error reports and transactions for the update

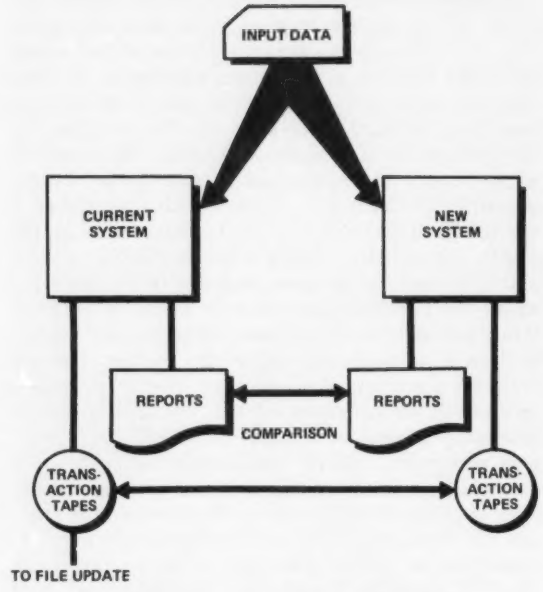

FIGURE 10.—Single-drug parallel testing, Sept 1975.

programs) are then compared between the two systems to identify any differences. These differences are then investigated to determine the need for changes in the new system. To minimize the legitimate variables between the two systems, this testing is accomplished initially without the introduction of new features; only as existing features are determined to be operational are new features added to the test environment.

As a result of this testing procedure, a definite date cannot be predicted for operational availability of the total redesign. Even as new features are added, care will be taken to ensure their operational status before involving the screeners. As an example, the introduction of terminals for remote data entry will not be accomplished in the field until the DPC has gained confidence in the programs, hardware, and procedural documentation through extensive use at its own facility.

CONCLUSION

Most of the changes discussed, while representing significantly more efficient capabilities than the current program design, will initially be somewhat transparent to the screeners who will still be submitting the same data on the same forms and handling data discrepancies in the same manner. This

will begin to change as the new features are introduced operationally with new forms and completion instructions or whole new methods of data entry. The changes do set the stage, however, for even more significant changes later, involving the master files and output reports which will handle the more sophisticated screening protocols being introduced into today's environment. It is anticipated that while any change is somewhat disturbing, the changes represented by the new forms or terminal utilization will be highly beneficial; they will eliminate many of the steps currently required to input the data within the current forms' limitations. Additionally, any reduction in calendar time required to advise screeners of input data discrepancies should assist both the NCI and the screeners in providing more accurate and timely data and thus should increase the value of the screening program.

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